Estrogenic Activity of a Diet to Estrogen Receptors -α and -β in an Experimental Animal

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Phytoestrogens, such as daidzein and genistein in plants are suspected as endocrine-disrupting chemicals (EDCs), because their chemical structures are similar to natural or synthetic estrogens, and have estrogenic activity *in vitro* and *in vivo*. An EDC study was carried out on the diets of various animals *in vivo*. However, many of these diets include phytoestrogens and may already possess estrogenic activity. In this study, we evaluated the estrogenic activity of phytoestrogens (such as daidzin, genistin, daidzein, genistein, coumestrol and equol) and the feed diets for experimental animals, such as fish, amphibians and reptiles, towards human estrogen receptors α (hER-α) and β (hER-β), and the genistein and daidzein content in these diets from HPLC analysis. Coumestrol showed the highest estrogenic activity for hER-α and -β in the –S9 test. Equol showed the highest estrogenic activity for hER-α in the +S9 test. The estrogenic activities of coumestrol, equol and genistein were approximately one hundred to two thousand times higher than that of daidzein. Many of these compounds showed higher compatibility with hER-β than with hER-α. A diet for fish from soybean was indicated to contain the highest amounts of genistein and daidzein. Moreover, this fish diet had the highest estrogenic activity for hER-α and -β. The estrogenic activity was found with hydrolysis by β-glucuronidase, showing higher compatibility with hER-β than with hER-α. In addition, correlation between the contents of genistein and estrogenic activities in the diets was found, with the exception of part of the diet. Therefore, this indicates that the genistein content contributes to the estrogenic activity of the diets. These results suggest that *in vivo* estrogenic activity might be caused by the diet provided to an experimental animal, indicating the necessity for more careful selection of the feeding diet and measurement of estrogenic substances when performing an *in vivo* screening assay for EDCs.

Key words —— endocrine-disrupting chemicals, estrogenic activity, yeast two-hybrid assay, phytoestrogen, feeding diet

INTRODUCTION

Recently, studies concerning the influence of endocrine-disrupting chemicals (EDCs) on humans and wildlife have been performed. Since these chemicals have similar structures to natural and synthetic hormones, these chemicals could accidentally bind to the hormone receptor, thereby inducing abnormal hormonal activities, and potentially causing disruption of metabolic systems due to the induction of drug-metabolizing enzymes and inhibition of enzymatic activity, disruption of the immune system, and carcinogenicity or genetic damage.[1)] Various *in vitro* screening assays have been developed to evaluate EDCs that may bind to sex hormone receptors. Screening assays such as the receptor-binding assay check the direct binding ability of the chemical with the receptor and the use of cultured cells such as yeast cells to which the receptor gene is inserted are frequently used. As for *in vivo* assays, the immature rat uterotrophic assay[2)] is used to determine estrogen and the hershberger assay[3)] to determine androgens are mainly used for mammals, and plasma and/or hepatic vitellogenin induction assay to determine estrogenicity have been used in fish[4)] and amphibians.[5)]

An EDC study was carried out on various ani-

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Table 1. Ingredients of the Diet for an Experimental Animal

Diet	Ingredients
Carp A	flour, defatted soybean, fish meal, alfalfa meal, shrimp meal, spirulina
Carp B	no data[a)]
Carp C	fish meal, flour, soybean cake and meal, yeast, salt, calcium phosphate
Carp D	Artemia
Trout A	fish meal, flour, soybean cake and meal, rice bran, yeast, vegetable oil
Trout B	no data[a)]
Medaka A	fish meal, cereal, yeast
Medaka B	fish meal, flour, soybean cake, sake lees, corn, shrimp meal, alfalfa meal, gluten meal, Spirulina, vitamin mix
Frog	Spirulina, Daphnia, fish liver, plankton, mussel
Turtle	no data[a)]
Alligator A	no data[a)]
Alligator B	Spirulina

a) Data concerning the ingredients could not be obtained.

mals *in vivo*, and the diet used for test animals was examined. However, many of these diets include the phytoestrogens (such as genistein and daidzein) and may already possess estrogenic activity. Therefore, phytoestrogens in the diet of experimental animals may affect the results of *in vivo* screening assays for EDCs. Although phytoestrogens are natural compounds drawing attention in Europe and the U.S. chiefly due to their health benefits, there is a report that in Australia, sheep fed diets with large amount of clover containing coumestrol increased the number of miscarriages.[6)] Furthermore, phytoestrogens such as coumestrol and equol have binding affinities for the estrogen receptor, higher than that of synthetic chemicals that are known to have estrogenic activity such as bisphenol A and nonylphenol.[7)] Therefore, the results of *in vivo* screening assays for EDCs may be seriously affected by phytoestrogens in the diet or estrogenic activity of the chemical, depending on the content level or the intensity of estrogenic activity.

This study aimed to elucidate the estrogenic activity of a commercial diet for experimental animals. Phytoestrogen content (genistein and daidzein) in the diet extract was measured by high-performance liquid chromatography (HPLC). Then the estrogenic activity of the typical phytoestrogens (genistein, daidzein, equol, coumsterol, genistin and daidzin) was measured with the two-hybrid assay using two types of yeast to which human estrogen receptors α (hER-α) and β (hER-β) have been incorporated. The phytoestrogens with activated metabolism using rat S9 liver were performed with similar procedures. The assay to determine the estrogenicity was also performed on extracts of commercial diets for fish, amphibians and reptiles using two types of yeast in order to evaluate the relationship between estrogenic activity and the phytoestrogen content of the feed diet.

MATERIALS AND METHODS

Chemical Analysis of Phytoestrogens in Experimental Animal Diets ——

Extraction and Preparation: Twelve animal diets, including fish, amphibian and reptile diets were used in the present study. Ingredients of the diets are shown in Table 1. Five grams of crushed sample was homogenized with 40 ml of methanol and 1 M-acetic acid buffer solution (9 : 1 volume ratio, pH 4.5). After homogenization, the sample was sonicated for 10 min in a 50 ml centrifuge tube. After shaking by hand for 10 min, the tube was centrifuged at 2000 rpm for 10 min. The supernatant was transferred to a 200 ml-measuring flask. A fresh batch of the methanol-acetic acid buffer solution was added to the precipitate that remained in the centrifuge tube and the operation was repeated. The extract was then adjusted to 200 ml with methanol. A portion (1 or 2 ml) of the extract was dried under nitrogen at room temperature. The dried extract was redissolved in 200 μl of purified water by vortex mixing. In the case of enzymatic hydrolysis, the extract was mixed with 1 ml of 0.5 M acetic acid buffer solution (pH 4.5) containing 2100 U β-glucuronidase and 1050 U arylsulfatase (Nippon Biotest Laboratories Inc., Tokyo, Japan). The reaction mixture was incubated overnight at 37°C. After the incubation, isoflavone present in the sample was extracted twice

with 5 ml diethyl ether followed by drying under nitrogen at room temperature. In the sample both with and without hydrolysis, the dried extract was redissolved in 200 μl methanol-acetonitrile-purified water mixture (2 : 1 : 3 volume ratio) (for measurement of genistein and daidzein), and 100% dimethylsulfoxide (DMSO, Wako Pure Chemical Industries, Ltd., Tokyo, Japan) (for measurement of estrogenic activity).

Measurement of Genistein and Daidzein: Five milligrams of genistein (Wako Pure Chemical Industries Ltd.) and daidzein (Fijicco Co. Ltd., Tokyo, Japan) were each dissolved in 10 ml of methanol and diluted to a concentration of 100 μg/ml to prepare standard solutions. Each standard solution was diluted sequentially to prepare solutions with concentrations of 0.1, 0.2, 0.5, 1.0, 2.5, 5.0 and 10 μg/ml. To prepare the samples for measurement, the sample was sonicated for 1 min. After centrifugation for 1 min at 10000 rpm using Ultrafree-MC (0.22 μm, Millipore, Tokyo, Japan), 20 μl of the elute was injected into the HPLC system, which was an LC 2000 Plus series HPLC manufactured by JASCO with a PEGASIL ODS analysis column (4.6 mm × 150 mm; manufactured by Senshu Kagaku, Tokyo, Japan). The mobile phases used were a mixture of methanol and acetonitrile (2 : 1) and 0.1% acetic acid employing a linear gradient with ultraviolet detection at 280 nm. The eluted peak was measured on the basis of the obtained peak area. In this system, the minimum detection limit was 0.5 μg/ml for genistein and daidzein. The recovery of both substances from the food was more than 90%. The coefficient of variation for the inter-assay was 2% or less (n = 3). The coefficient of variation for the intra-assay was 5% or less (n = 3).

Yeast Two-Hybrid Assay ——

Chemicals: Estradiol-17β (β-E2, Sigma, St. Louis, MO, U.S.A.) and *t*-stilbene (T-S, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) were used as the positive control of –S9 and +S9 tests, respectively. In the yeast two-hybrid assay, genistein (Wako Pure Chemical Industries Ltd.), daidzein (Fijicco Co. Ltd.), coumestrol (Funakoshi Co., Ltd., Tokyo, Japan), equol (Funakoshi Co., Ltd.), genistin (Sigma) and daidzin (Funakoshi Co., Ltd.) were used. In the case of genistin and daidzin, enzyme-processed samples with β-glucuronidase were also prepared. These samples were dissolved in DMSO, dispensed into glass sample bottles in small doses, and preserved in a frozen state at –20°C.

Estrogen Agonist Test Using the Yeast Two-Hybrid Assay: The estrogenic activities of phytoestrogens and commercial diets were measured by a yeast two-hybrid assay as described by Shiraishi *et al*.[8] The activities of phytoestrogens were also measured both with and without possible metabolic activation by rat liver S9 preparation (Kikkoman Company, Japan). The sample for metabolic activation was performed after incubating the sample with rat liver S9 mix for 1 hr at 37°C. This system was performed with yeast cells (*Saccharomyces cervisiae* Y190) prepared by incorporating the human estrogen receptor α and β, expression plasmid of the coactivator TIF2, and β-galactosidase expression reporter in the yeast two-hybrid assay.[9]

Aliquots of sample solutions (20 μl) were incubated (30°C, 4 hr) with yeast cells in a 96-well microplate (SUMILON, Sumitomo Bakelite, Japan) that had been preincubated (30°C, overnight) in modified synthetic dropout (SD) medium lacking tryptophan and leucine. A mix solution for inducing chemiluminesence and for enzymatic digestion (Zymolyase 20T) was added followed by a light emission accelerator solution. The chemiluminesence produced by released β-galactosidase was measured with a 96-well plate luminometer (Luminescencer-JNR AB2100, ATTO Bio-Instrument, Tokyo, Japan). Agonist activity was recorded as the $EC_{\times 10}$ which was defined as the concentration of test solution producing a chemiluminescent signal 10 × that of the blank control. The inverse of the obtained $EC_{\times 10}$ values of β-E2 and T-S were set to 100. Similar procedures were taken for other samples to calculate the β-E2 relative activity.

RESULTS AND DISCUSSION

Genistein and Daidzein Content in the Diet

In order to evaluate the genistein and daidzein content in the feed diet, the genistin and daidzin present as glycosides were hydrolyzed with β-glucuronidase and sulfatase, and the total amount of genistein and daidzein content found in the feed diet are shown in Table 2. The carp A diet contained the largest amount of genistein, which was 237.2 ± 7.1 μg/g, followed by those of alligator B diet (186.1 ± 1.3 μg/g), and the carp B diet (141.6 ± 5.3 μg/g). Similarly, the carp A diet contained the largest total amount of daidzein (175.8 ± 2.6 μg/g), followed by those of the alligator B diet (140.0 ± 1.4 μg/g), and the carp B diet (107.2 ±

2.6 μg/g).

Tsukamoto *et al*. reported that genistin and daidzin, which are glycosides of genistein and daidzein, were present in wholegrain soybean at levels of 1.1 ± 0.6 to 15.0 ± 1.5 mg/100 g and 0.6 ± 0.1 to 10.2 ± 1.8 mg/100 g, respectively. Furthermore, the following 12 isoflavone types have been identified in soybean: 3 types of aglycon (daidzein, genistein and glycitein), their glycosides (daidzin, genistin and glycitin), and their malonylized glycosides and acetylized glycosides.[10] In this study, we hydrolyzed the genistin and daidzin glycosides in the diet in order to measure the total amount of genistein and daidzein. Therefore, the amount of glycosides without hydrolysis and the content of genistin and daidzin are unknown. However, no estrogenicities were found without hydrolysis in the diets as a result of the yeast two-hybrid assay for hER-α, but these estrogenicities were found after hydrolysis (Table 3). Therefore, it is possible that these phytoestrogens were present in the form of genistin and daidzin, which were hydrolyzed and then detected as genistein and daidzein. Court *et al*.[11] measured the genistein and daidzein content in different types of commercial cat food. Genistein and daidzein were detected in 24 out of 42 types that were surveyed, within a concentration range of 1 to 163 μg/g. The carp A diet, which was found with the highest levels of genistein and daidzein in this study, contained defatted soybean and higher levels of genistein and daidzein than those in the commercial cat food. Furthermore, genistein and daidzein were detected in 10 out of 12 types of diet surveyed in this study. This indicated that most types of commercial feeding diet for experimental animals might contain phytoestrogens.

Pelissero *et al*. reported that blood vitellogenin levels of Siberian sturgeon *Acipenser baeri* (*A. baeri*), that were dosed with 0.2 mg of genistein per 20 g body weight, increased to 213 ± 56 μg/ml (control group: 0.1 μg/ml or less).[12] If carp A diet with

Table 2. Contents of Genistein and Daidzein in an Animal Feed Determined by HPLC Analysis

Diet	Total genistein (μg/g)	Total daidzein (μg/g)
Carp A	237.2 ± 7.1	175.8 ± 2.6
Carp B	141.6 ± 5.3	107.2 ± 2.6
Carp C	67.5 ± 0.3	50.2 ± 0.9
Carp D	N.D.	N.D.
Trout A	41.6 ± 2.2	30.5 ± 0.4
Trout B	50.2 ± 0.7	33.2 ± 0.6
Medaka A	9.3 ± 0.1	33.2 ± 0.1
Medaka B	58.5 ± 0.6	37.3 ± 0.2
Frog	N.D.	—
Turtle	31.4 ± 1.2	33.6 ± 1.6
Alligator A	26.7 ± 0.4	19.9 ± 0.3
Alligator B	186.1 ± 1.3	140.0 ± 1.4

N.D., Not Detected (< 0.8 μg/g); —, Not Determined. The data represent the mean and standard deviation ($n = 3$).

Table 3. Estrogenic Activities of an Animal Feed Diet for hER-α and -β Using the Yeast Two-Hybrid Assay

Diet	Relative estrogenic activity[a]			
	hER-α		hER-β	
	Without	With	Without	With
β-E2	100	100	100	100
Carp A	—	1.4×10^{-5}	5.4×10^{-6}	1.6×10^{-4}
Carp B	—	9.6×10^{-6}	4.0×10^{-6}	7.6×10^{-5}
Carp C	—	4.9×10^{-6}	—	3.5×10^{-5}
Carp D	—	—	—	—
Trout A	—	1.6×10^{-6}	—	1.9×10^{-5}
Trout B	—	2.7×10^{-6}	—	3.0×10^{-5}
Medaka A	—	—	—	—
Medaka B	—	3.6×10^{-6}	—	2.1×10^{-5}
Frog	—	—	—	—
Turtle	—	—	—	7.7×10^{-6}
Alligator A	—	—	—	1.2×10^{-5}
Alligator B	—	8.4×10^{-6}	2.4×10^{-6}	6.1×10^{-5}

a) The estrogenic activities of the diets were record as the EC ×10 which was defined as the concentration (weight base) of test diet producing a chemiluminescent signal 10× that of the blank control. Data of agonist test were calculated as relative estrogenicity of β-E2; 100. Diets with or without hydrolysis are also shown. β-E2: estradiol-17β. —, Not Detected. The data represented are the mean ($n = 4$).

the highest genistein level (237.2 ± 7.1 μg/g) was fed to *A. baeri*, at 3% per 20 g of body weight, it was calculated that approx. 0.14 mg of genistein is given to *A. baeri*. This is a sufficient quantity to cause induction of blood vitellogenin. From these facts, if the screening assay for EDCs is performed using blood vitellogenin production as a biomarker, the estrogenic activity of the test chemical may be seriously affected by the estrogenic activity of the genistein and daidzein content of the carp A diet, and the estrogenic activity may not be detected depending on the intensity of estrogenic activity of the test chemical.

Generally in the human body, the absorbed isoflavone is subjected to glucuronate and sulfate conjugation, and migrates into the blood, most of which is excreted in the urine. It is also known that part of the isoflavone in blood migrates to the intestinum tenue *via* bile and is de-conjugated by the intestinal flora and enters into the enterohepatic circulation.[13] On the other hand, mammals such as mouse, rat and animals including fish, reptiles and amphibians do not have identical absorption, metabolism and excretory mechanisms as those of humans. While the free and sulfate-conjugated phytoestrogens have *in vivo* estrogenic activity, it is considered that their quantity is much smaller than glucuronate-conjugated phytoestrogen.[14] It will be necessary to elucidate the *in vivo* dynamics of phytoestrogen in each species, and to establish a method to measure the free and sulfate-conjutaged phytoestrogens separately. Furthermore, there are reports that genistein and daidzein have an aromatase inhibitory effect,[15] and that genistein and daidzein stimulate the production of sex hormone-binding globulin (SHBG), which causes a reduction of activated estrogen in the liver.[16] Therefore, when performing an *in vivo* screening assay of EDCs, it will be necessary to consider the basic physiological function of the phytoestrogen in the body of the subject as well as measuring the quantity of phytoestrogens such as genistein and daidzein.

Estrogenic Activity of Phytoestrogens

The assay to determine the estrogenic activity in the previous study was performed with phytoestrogens, which are not activated metabolically by rat S9 liver preparation. However, few assays have been performed for substances that are activated metabolically. Phytoestrogens taken into the living organism are metabolized by drug-metabolizing enzymes of the liver, *etc*., and therefore it is important to assess the estrogenic activity of metabolic products when considering the effect of phytoestrogen on living organisms. In this study, we used the yeast two-hybrid assay and assessed the estrogenic activity of phytoestrogens, such as genistein, daidzein, coumestrol, equol, genistin and daidzin, with –S9 (Fig. 1) and +S9 tests (Fig. 2). A summary of the estrogenic activity of phytoestrogens is shown in Table 4.

In the –S9 test, coumestrol showed the highest estrogenic activity for hER-α and -β. The relative estrogenic activities of coumestrol for hER-α and -β were estimated to be 9.7×10^{-1} and 2.1, respectively. These values were 1/100 and 1/50 of the relative activity of the positive control substance β-E2 100, respectively. On the other hand, in the +S9 test, no estrogenic activity of coumestrol for hER-α and -β was detected. The relative estrogenic activities of genistin and daidzin for hER-α in the –S9 test were 3.4×10^{-4} and 3.2×10^{-4}, respectively. With hydrolysis by β-glucuronidase, these values changed to 7.8×10^{-2} and 5.4×10^{-4}, respectively. These values were similar to those of aglycon, genistein (relative activity: 7.6×10^{-2}) and daidzein (relative activity: 5.4×10^{-4}). The relative estrogenic activities of genistin and daidzin for hER-β were 4.4×10^{-3} and 3.3×10^{-4}, respectively. These values changed to 3.9×10^{-1} and 1.9×10^{-3}, respectively through hydrolysis by β-glucuronidase, which also showed similar values as those of genistein (relative activity: 4.7×10^{-1}) and daidzein (relative activity: 1.9×10^{-3}). These results supported the preceding reports that the glycosides, genistin and daidzin, are hydrolyzed by enteric bacteria, by β-glucuronidase in living organisms, and metabolized to genistein and daidzein respectively.[17] The estrogenic activity levels of genistin and daidzin for hER-β were three to five times higher than those for hER-α. In this study, although the glycosides, genistin and daidzin, showed the low estrogenic activity for hER-α and -β, the permeability of glycosides to the yeast cell wall is unknown. It is considered that yeast two-hybrid system basically shows lower responsiveness to estrogenic substances than that of mammalian cell system because of low permeability of yeast cell wall. Therefore, glycosides might not be able to accessible to nuclear receptor due to low permeability. It will be necessary to assess the permeability of glycoside to yeast cell wall in the future study. In the +S9 test, the relative activity of genistin for hER-α was 7.3×10^{-4}, but no estrogenic activity was detected with daidzin. With hydrolysis by β-glucu-

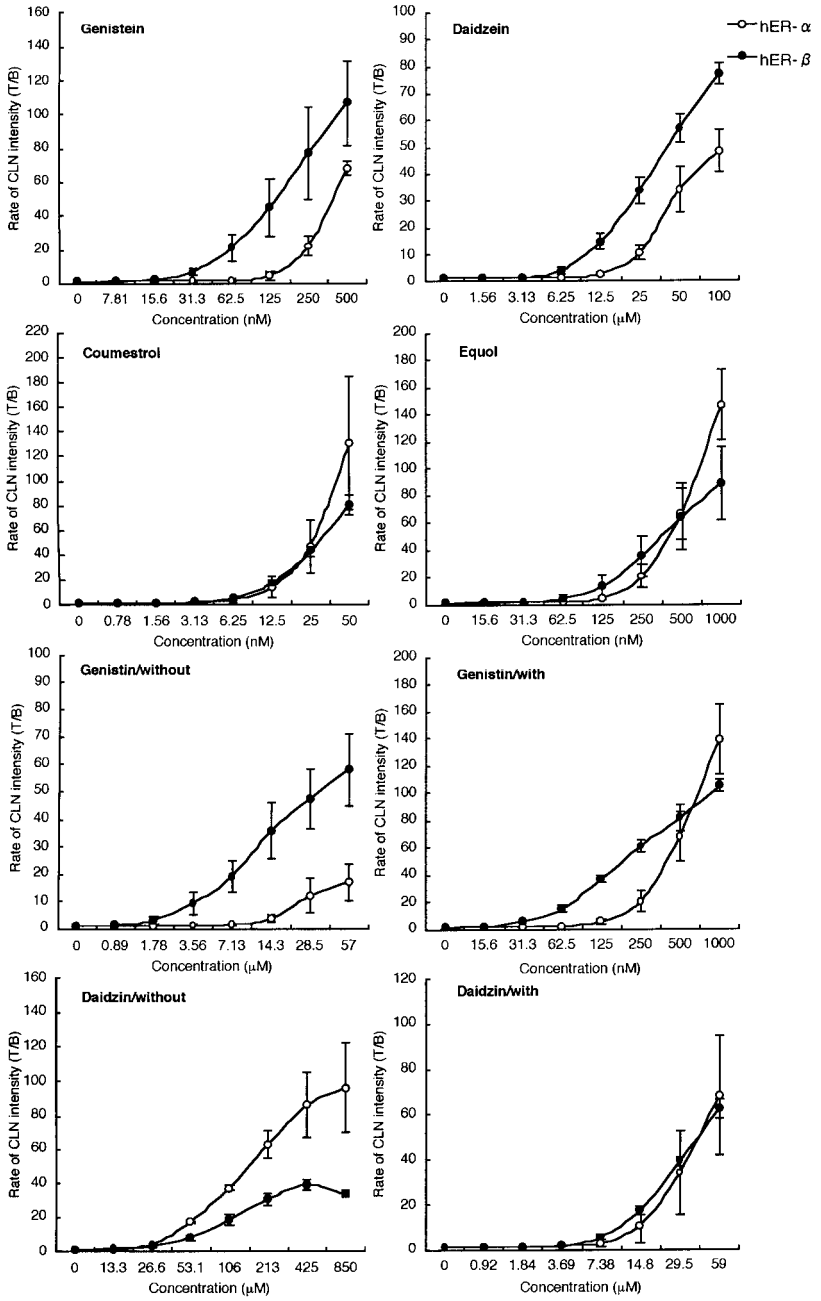

Fig. 1. Dose–Response Curves for Genistein, Daidzein, Coumestrol, Equol, Genistin with and without Hydrolysis, and Daidzin with and without Hydrolysis Using the Agonist (–S9) Test for the Yeast Two-Hybrid Assay for Estrogen Receptor α (○) and β (●)

The values were represented as the ratio of chemiluminescence (CLN) intensity (T/B) of β-galactosidase. Data represented are the means and standard deviation ($n = 4$).

ronidase, no activity was detected with genistin, but daidzin (relative activity: 4.8×10^{-4}) showed a similar level of relative activity as that of daidzein (relative activity: 3.6×10^{-4}). On the other hand, the relative activity level of genistin for hER-β was 3.2×10^{-3}, but no estrogenic activity was detected with daidzin. Similarly, with hydrolysis by β-glucuronidase, genistin did not show any estrogenic activity for hER-β, but daidzin (relative activity: 1.5×10^{-3}) showed a similar level of relative activity as that of daidzein (relative activity: 1×10^{-3}). In the –S9 test, the relative activity levels of equol for hER-α and -β were estimated to be 7.3×10^{-2} and 1.7×10^{-1}, respectively. In the +S9 test, however, the relative activity of equol for hER-α was 1.9×10^{-2}, but no estrogenic activity was detected for hER-β.

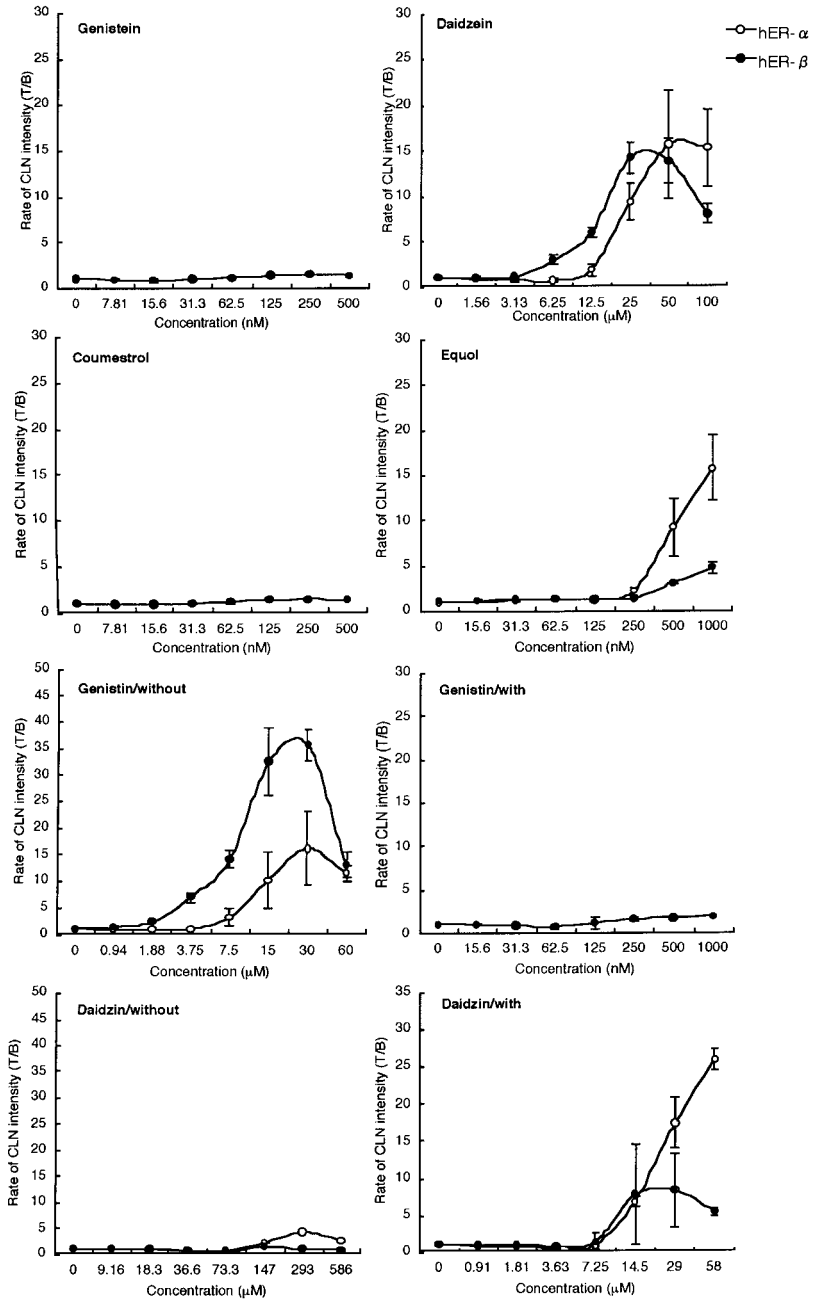

Fig. 2. Dose–Response Curves for Genistein, Daidzein, Coumestrol, Equol, Genistin with and without Hydrolysis, and Daidzin with and without Hydrolysis Using the Agonist (+S9) Test for the Yeast Two-Hybrid Assay for Estrogen Receptor α (○) and β (●)

The values were represented as the ratio of chemiluminescence (CLN) intensity (T/B) of β-galactosidase. Data represented are the means and standard deviation ($n = 4$).

These results suggest that the estrogenic activity of most phytoestrogens decreased due to metabolism in the +S9 test using the rat S9 liver preparation. Morito *et al.*[17] performed an estrogenic activity test of daidzein, genistein and equol for hER-α and -β with the yeast two-hybrid assay. They reported that in the –S9 test equol demonstrated the highest gene expression ability for both receptors, particularly for hER-α. They also reported that the strengths of gene expression ability of these substances are in the order of daidzein < genistein < equol. In this study, however, those for hER-α and -β were in the order of daidzein < equol < genistein < coumestrol. Estrogen activities of equol and genistein were found at similar concentrations, seemingly almost the same as the result of the test conducted by Morito *et al*. In

Table 4. Estrogenic Activities of Phytoestrogens for hER-α and -β Using the Yeast Two-Hybrid Assay

Compounds	Relative estrogenic activity[a]			
	hER-α		hER-β	
	−S9	+S9	−S9	+S9
β-E2	100	—	100	—
T-S	—	8.2×10^{-3} (100)	—	8.1×10^{-3} (100)
Genistein	7.6×10^{-2}	—	4.7×10^{-1}	—
Daidzein	5.4×10^{-4}	3.6×10^{-4} (4.4)	1.9×10^{-3}	1.0×10^{-3} (12.3)
Coumestrol	9.7×10^{-1}	—	2.1	—
Equol	7.3×10^{-2}	1.9×10^{-2} (232)	1.7×10^{-1}	—
Genistin/without	3.4×10^{-4}	7.3×10^{-4} (8.9)	4.4×10^{-3}	3.2×10^{-3} (39.5)
Genistin/with	7.8×10^{-2}	—	3.9×10^{-1}	—
Daidzin/without	3.2×10^{-4}	—	3.3×10^{-4}	—
Daidzin/with	5.4×10^{-4}	4.8×10^{-4} (5.9)	1.9×10^{-3}	1.5×10^{-3} (18.5)

a) The estrogenic activities of phytoestrogens were record as the EC $\times 10$ which was defined as the concentration of test solution producing a chemiluminescent signal $10\times$ that of the blank control. Data of −S9 test were calculated as relative estrogenicity of β-E2; 100, and +S9 test were calculated as relative estrogenicity of T-S; 100. Genistin and daidzin with or without hydrolysis are also shown. β-E2: estradiol-17β, T-S: *trans*-stilbene. —, Not Detected. The data represents the mean ($n = 4$).

our study, it was re-confirmed that coumestrol, equol and genistein have higher estrogenic activities than daidzein. When performing an *in vitro* assessment for estrogenic activity of a chemical substance, it will be necessary to consider metabolism with S9 liver.

Kuiper *et al*. evaluated the estrogenic activity of genistein, daidzein, bisphenol A and nonylphenol for hER-α and -β. They reported that the binding affinities of these substances for hER-α were 4, 0.1, 0.05 and 0.01, respectively whilst that for β-E2 was set to 100, and the binding affinities of these substances for hER-β were 87, 0.5, 0.09 and 0.01, respectively.[18] These results strongly suggest that it is necessary to assess the contents of phytoestrogens such as genistein and daidzein in the feed diet when performing an *in vivo* screening assay for EDCs. Moreover, it will also be necessary to consider and assess the interaction between the test chemicals and phytoestrogen.

Estrogenic Activity of the Feeding Diet

Phytoestrogens (*e.g*. genistein and daidzein) in the feed diet for experimental animals may affect the *in vivo* screening assay for EDCs. In this study, we measured the estrogenic activity of diets for hER-α and -β (Table 3). Except for some diets, no estrogenic activity for hER-α and -β was found in the diets without hydrolysis by β-glucuronidase. Furthermore, the estrogenic activity increased with hydrolysis by β-glucuronidase. The carp A diet containing unfatted soybean showed the highest estrogenic activity tested in this study, and the relative estrogenic activity levels for hER-α and -β were 1.4×10^{-5} and 1.6×10^{-4}, respectively. This diet contained the largest content of genistein and daidzein among the diets tested in this study. Since the estrogenic activities of genistin and daidzin increased after hydrolysis (Table 4), it suggested that genistin and daidzin in the carp A diet changed into genistein and daidzein as a result of hydrolysis, and that these substances contributed to the increase in estrogenic activity of the carp A diet.

Similar to phytoestrogens, concerning the estrogenic activity of the feed diet, a higher estrogenic activity was found for hER-β than for hER-α. Generally it is known that phytoestrogens have a higher affinity for the β receptor than for the α receptor.[17] In this study, all diets that had estrogenic activity showed higher affinity for the β receptor than for the α receptor. Therefore, it indicated that phytoestrogens were mainly substances that contributed to the estrogenic activity of the diet. Recently, hER-β has been isolated, but little is known about its physiological effects. However, besides its physiological effects being similar to those of hER-α, different physiological effects due to differences in the control ability of target genes and expressional domain may be considered. In this study, phytoestrogen and different types of feed diet showed high estrogenic activity for hER-β. This means there is a possibility of different physiological effects during the *in vivo* screening of EDCs if feed diets containing phytoestrogen are used. It is necessary to study in

detail the basic physiological effect of such a diet and the compound effects of the diet and test chemical. Matthews *et al*.[19] demonstrated the genistein affinity for the estrogen receptor of human, mouse, chicken, green anole and rainbow trout. They reported that the estrogen receptor of green anole showed the highest affinity compared with those of the other species. The Environment Agency, Japan measured the binding ability of nonylphenol (mixture) and 4-*t*-octylphenol with the medaka and human estrogen receptor (α) ligand-binding domain expressed through *Escherichia coli* (*E. coli*) by the competitive binding test with [^{3}H] β-E2.[20] Nonylphenol (mixture) and 4-*t*-octylphenol showed a concentration-dependent affinity for the medaka estrogen receptor (α). Their relative affinities were approximately 1/10 and 1/5 of those of β-E2 respectively, and suggested that they have stronger affinity than for the human estrogen receptor (α) (about 1/2000 to 1/3000 of those of β-E2). In a similar test for the β receptor, nonylphenol showed the relative binding affinity of approximately 1/110 against that of β-E2, which is approximately 30 times higher than that of the human estrogen receptor. In this study, we used hER-α and -β to test the estrogenic activity of the feed diet for fish, reptiles and amphibians. The affinity of phytoestrogen for the intrinsic receptor may depend on the species. We will need to develop a test method for estrogenic activity to which the receptor of each species is incorporated.

Ishibashi *et al*.[21] reported that the effects of nonylphenol on the production of plasma vitellogenin and steroid hormones were measured in goldfish *Carassius auratus* fed on a diet with a low or high content of phytoestrogens. The results suggest that nonylphenol has estrogenic activity in male goldfish, but it is possible that estrogenic substances such as phytoestrogens when present in high concentrations could interact with nonylphenol in binding estrogen receptors, with the possibility of masking estrogenic effects of nonylphenol when evaluating EDCs using plasma vitellogenin production as a biomarker in fish. It will be necessary to measure the phytoestrogen and the overall estrogenic activity of the feed diet before assessing EDCs. Phytoestrogens bind to the estrogen receptor, accelerate gene activation, and show concentration-dependent effects similar to tamoxifen. Therefore, phytoestrogens may have estrogenic or antagonistic effects. In this study, if the assessed feeding diet is taken into the living organism, it is not known whether the phytoestrogen works synergistically or antagonistically with the estrogen and other test agent possessing estrogenic effects. There may be an interaction between the phytoestrogen and these estrogenic substances in the living organism, and therefore we must elucidate their behavioral mechanism.

Relationship between Estrogenic Activity and Phytoestrogen in the Diet

We evaluated the estrogenic activity level of the feeding diet for experimental animals found by the yeast two-hybrid assay and the total content of genistein and daidzein analysed by HPLC to study the relationship between estrogenic activity and the phytoestrogen content. We used the $EC_{\times 10}$ values of β-E2, found by the yeast two-hybrid assay for genistein and daidzein to calculate the β-E2 conversion of total genistein and total daidzein contents in the diet as measured by HPLC, respectively. We used the sum of these values as the β-E2 conversion of the phytoestrogen (genistein and daidzein) content (Fig. 3-X axis). On the other hand, by using the $EC_{\times 10}$ values of β-E2, genistein and daidzein found by the yeast two-hybrid method and the $EC_{\times 10}$ value of different types of diet, we calculated the estrogenic activity β-E2 conversion by the yeast two-hybrid assay (Fig. 3-Y axis).

In most diets (for hER-α and -β), positive correlation was found between the β-E2 conversion from the phytoestrogen content and the estrogenic activity of β-E2 conversion by the yeast two-hybrid assay ($p < 0.001$). Therefore, it is strongly suggested that genistein and daidzein, which are phytoestrogens, largely contribute to the estrogenic activity of these diets for hER-α and -β. However, some diets were outside the regression curve in the relationship between the β-E2 conversion of the phytoestrogen content in the diet and the estrogenic activity for β-E2 conversion by the yeast two-hybrid assay. Pelissero *et al*. reported that 9.35 ± 3.5 ng/g of β-E2 and 6.15 ± 1.9 ng/g of estrone (E1) in a commercial diet for fish were detected, respectively. These hormone levels were involved in the production of blood vitellogenin in Siberian sturgeon. They also suggested a high possibility of β-E2 and E1 in the ingredients of biological origin such as fish powder.[22] Furthermore, the plants used as raw materials for the feeding diet may contain other substances with anti-estrogenic and (anti) androgenic effects. It is necessary to identify such (anti) estrogenic and (anti) androgenic substances in the feed diet. In the future, instrumental analysis of known estrogenic

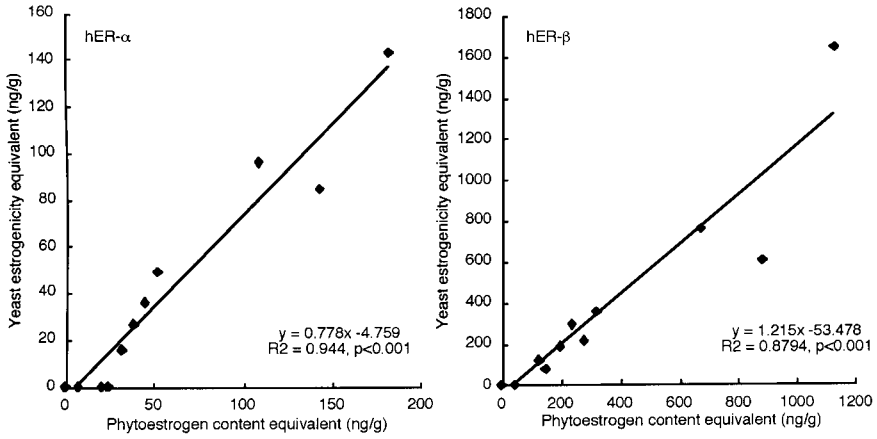

Fig. 3. Relationship between the Estrogenicity Equivalent from the Estrogenic Activities for the Human Estrogen Receptor-α (hER-α) and β (hER-β) Using the Yeast Two-Hybrid Assay and the Estrogenicity Equivalent from the Contents of Genistein and Daidzein Using HPLC Analysis of a Diet for an Experimental Animal

substances contained in the feed diet will not be sufficient, but the effect of other substances should be considered. We expect that the measurement of total estrogenic activity in the diet by the yeast two-hybrid assay as was carried out in this study will be performed in future studies, as well as more careful selection of feed diet and the development of new diets which are low in phytoestrogens.

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