

The Potential Contribution of Phytoestrogens and Organochlorine Pesticides in an Experimental Fish Diet to Estrogenic Activity

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Phytoestrogens and organochlorine pesticides in the diet of laboratory animals are a possible source of interference in bioassays that assess estrogenic activity. In the present study, we investigated the levels of dietary phytoestrogens, organochlorine pesticides and the estrogenic activity of various diets for an experimental fish and discuss the potential contribution of these substances to estrogenic activity, in comparison with those used in previous studies. After hydrolysis with β -glucuronidase, genistein and daidzein were detected in all of the diets, and there were no significant differences in the contents of these substances among present and previous investigations. In addition, organochlorine pesticides, such as hexachlorobenzene (HCB), β -benzene hexachloride (β -BHC), γ -BHC, *trans*-nonachlor, and/or endrin, were detected in most fish diets. All of these diets exhibited higher levels of activation of β estrogen receptors than with α estrogen receptors in an *in vitro* yeast-based bioassay. These results indicate that phytoestrogens, such as genistein and daidzein, were the main substances contributing to the estrogenic activity of the diet. Moreover, some

diets may exert estrogenic activity in *in vivo* tests, indicating the necessity for more careful selection of the feeding diet and measurement of estrogenic substances when performing routine screening assays for endocrine-disrupting chemicals.

Key words — endocrine-disrupting chemical, phytoestrogen, organochlorine pesticide, estrogenic activity, fish diet

INTRODUCTION

The public and scientific concerns of endocrine-disrupting chemicals (EDCs) present in the human diet and in the environment, and their potency to disrupt the normal hormonal status in humans and wildlife, have become a high-profile international issue.^{1–3} Many of these EDCs have similar structures to natural and synthetic hormones, and can accidentally bind to steroid hormone receptors, such as estrogen, androgen and thyroid receptors. In order to regulate and minimize the release of hormonally active agents into the environment, the Organization for Economic Cooperation and Development (OECD) initiated a high-priority activity in 1997 to develop new and revised guidelines for screening and testing potential EDCs.⁴ For the screening of estrogenic substances, many *in vitro* and *in vivo* assays were developed and conducted: MCF-7 cell proliferation assay (E-screen assay),⁵ yeast estrogen-screen assay (YES assay),⁶ yeast two-hybrid assay,^{7,8} rat uterotrophic assay⁹ and the vitellogenin (Vg, egg yolk protein precursor) production assay.^{10,11} Since the estrogenic effect of test chemicals on individual animals is examined with *in vivo* assays, fish are often used as a laboratory test animal and also as representatives of aquatic animals. During these *in vivo* assays, fish are often fed on commercial diets.

Phytoestrogens in the laboratory diet are a possible source of interference with bioassays that assess estrogenic activity. Phytoestrogens and derivatives, such as genistein, daidzein, coumestrol and equol, could potentially disturb the reproductive function in mammals.^{12,13} Many of these compounds may act as estrogen receptor agonists, binding to estrogen receptors and stimulating estrogen-responsive gene expression,^{14,15} or may antagonize the binding of endogenous estrogen to its receptors and exert anti-estrogenic physiological effects.¹⁶ For

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example, phytoestrogens have been shown to elicit statistically significant increases in uterine weights at high doses in the uterotrophic assay.¹⁷⁾ Our previous study also demonstrated the effects of a phytoestrogen-enriched diet on the production of plasma Vg and steroid hormones in male goldfish (*Carassius auratus*).¹⁸⁾ Phytoestrogens such as coumestrol and equol exhibit higher binding affinities for the estrogen receptor than those of known xenoestrogens such as bisphenol A and nonylphenol.¹⁹⁾

Persistent organic pollutants, some with known potential as EDCs, are adventitious compounds present in a variety of diets.²⁰⁾ To date, many of these chemicals, such as β -benzene hexachloride (β -BHC), *p,p'*-dichlorodiphenyl dichloroethylene (DDE), *o,p'*-dichlorodiphenyl trichloroethane (DDT) and endrin, have been reported as estrogenic pesticides.²¹⁾ The studies have also revealed anti-androgenic pesticides, such as vinclozolin and *p,p'*-DDE.²¹⁾ Our previous study demonstrated that organochlorine pesticides, such as hexachlorobenzene (HCB), β -BHC, and/or γ -BHC, could be detected in fish diets.²²⁾ Although there are a few studies concerning adventitious contaminants, such as phytoestrogens and organochlorine pesticides,^{22,23)} the potential contribution of these chemicals in unpurified commercial fish diets is unknown. It is possible that estrogenic substances in the fish diet compete with test chemicals in binding estrogen receptors in the *in vivo* estrogen assay and may affect the estimation of the estrogenic activity of test chemicals.

In the present study, we assessed the levels of dietary phytoestrogens, organochlorine pesticides and the estrogenic activity of various diets for an experimental fish and discuss the potential contribution of these substances to estrogenic activity compared with diets tested in previous studies.

MATERIALS AND METHODS

Measurement of Genistein and Daidzein — Six diets for teleost fish (such as mullet and sea bream) used in the present study, are commercial available in Korea, respectively. The extraction of phytoestrogens (genistein and daidzein) and estrogenic substances from fish diets was performed by previous methods as described by Miyahara *et al.*²³⁾ Briefly, 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. Then the extract was adjusted to 200 ml with methanol. A portion (1 or 2 ml) of the extract was dried under nitrogen at room temperature. In the case of enzymatic hydrolysis, the dried extract was redissolved in 200 μ l of purified water by vortex mixing. This solution 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, phytoestrogens and/or estrogenic substances 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 μ l 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.

The measurement of genistein and daidzein were determined by a high-performance liquid chromatography (HPLC) as described by Miyahara *et al.*²³⁾ 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 μ g/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 μ g/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 μ m, Millipore, Tokyo, Japan), 20 μ l 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 \times 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 assay, the minimum detection limit was 0.5 μ g/ml for genistein

and daidzein. In this system, the recovery of both substances from the fish diet was more than 90%. The coefficient of variation for the inter-assay was 2% or less ($n = 3$), and the coefficient of variation for the intra-assay was 5% or less ($n = 3$).²³⁾

Measurement of Organochlorine Pesticides — The measurement of organochlorine pesticides was performed by previous methods as described by Inudo *et al.*²²⁾ Briefly, three hundreds milligrams of crushed sample was extracted with 5 ml of *n*-hexane by mild sonication for 10 min, and centrifuged at 2000 rpm for 10 min. The supernatant was transferred into a 10 ml tube. On the residue, the above operations were repeated once. The supernatants thus obtained were combined and treated with a Sep-Pak Florisil cartridge column (500 mg, Waters, U.S.A.). The eluant was added to 1 ml of dodecane and 200 μ l of 1-nitropyrene (1 mg/l) internal standard solution, concentrated to 1 ml with a Syncore Polyvap R-24, and then analyzed for the organochlorine pesticides profiles by a gas chromatography (GC) with electron capture detection (ECD), as described below.

A GC/ECD was performed on a Agilent 6890 GC system (Agilent Technologies, U.S.A.) using a Hewlett-Packard HP-5 capillary column (30 m \times 0.32 mm, 0.25 μ m film thickness). The temperature program was as follow: 50°C for 1.5 min, 30°C/min to 200°C, held for 3 min, 20°C/min to 260°C, and 4°C/min to 280°C, held for 2 min. Injector and detector temperatures were at 200 and 300°C, respectively. Helium was used as carrier gas at 47 cm/sec. Injection volume was 2 μ l. The following organochlorine pesticides were screened for and routinely detected in the sample: HCB, β -BHC, γ -BHC, vinclozolin, *p,p'*-DDE, *trans*-nonachlor (*t*-nonachlor), endrin and *o,p'*-DDT.

Measurement of Estrogenic Activity — The estrogenic activities of fish diets were measured by a yeast two-hybrid assay as described by Miyahara *et al.*²³⁾ and Shiraishi *et al.*⁸⁾ This system was performed with yeast cells (*Saccharomyces cerevisiae* Y190) prepared by incorporating the human estrogen receptor α (ER α) and β (ER β), an expression plasmid of the coactivator transcription intermediary factor 2 (TIF2), and a β -galactosidase expression reporter in the yeast two-hybrid system.⁷⁾

Aliquots of sample solutions (20 μ l) 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 medium lacking tryptophan and leucine. A mix solution for inducing

chemiluminescence and for enzymatic digestion (Zymolyase 20T, Seikagaku, Corp., Tokyo, Japan) was added followed by a light emission accelerator solution. The chemiluminescence produced by released β -galactosidase was measured with a 96-well plate luminometer (Luminescencer-JNR AB2100, ATTO Bio-Instrument, Tokyo, Japan). Estrogenic activity was recorded as the EC_{x10} which was defined as the concentration (weight base) of test solution producing a chemiluminescent signal 10 \times that of the blank control. As the inverse of the obtained EC_{x10} values of estradiol-17 β (E2, Sigma, St. Louis, MO, U.S.A.), similar procedures were taken for other samples to calculate the E2 equivalent.

In addition, we evaluated the estrogenic activity level of the feeding diet for experimental fish 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.²³⁾ We used the EC_{x10} values of E2, found by the yeast two-hybrid assay for genistein and daidzein to calculate the 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 E2 conversion of the phytoestrogen (genistein and daidzein) content. On the other hand, by using the EC_{x10} values of E2, genistein and daidzein found by the yeast two-hybrid method and the EC_{x10} value of different types of diet, we calculated the estrogenic activity E2 conversion by the yeast two-hybrid assay.

RESULTS

Phytoestrogen Levels

In order to assess the genistein and daidzein content in the fish diet from Korea, genistin and daidzin present as glycosides were hydrolyzed with β -glucuronidase and sulfatase. The total amounts of genistein and daidzein in the fish diet tested in this study are shown in Table 1. The No. 3 diet contained the largest amount of genistein at 118.9 ± 1.2 μ g/g, followed by the No. 2 diet (115.7 ± 2.0 μ g/g), No. 1 diet (79.9 ± 3.7 μ g/g), No. 5 diet (59.2 ± 1.9 μ g/g), No. 6 diet (58.2 ± 0.5 μ g/g) and No. 4 diet (39.7 ± 0.5 μ g/g). Similarly, No. 3 diet contained the largest total amount of daidzein (81.3 ± 0.8 μ g/g), followed by the No. 2 diet (78.4 ± 1.1 μ g/g), No. 1 diet (60.0 ± 2.1 μ g/g), No. 6 diet (42.1 ± 0.8 μ g/g), No. 5 diet (40.1 ± 0.9 μ g/g) and No. 4 diet (28.7 ± 0.3 μ g/g). From a comparison of phytoestrogen levels in fish

diets from present and previous investigations,^{22,23} there were no significant differences in the genistein or daidzein contents ($p > 0.05$, Fisher'PLSD Post-hoc test) (Fig. 1).

Organochlorine Pesticide Levels

The organochlorine pesticide profiles in the fish diet samples are shown in Table 2. HCB, β -BHC and γ -BHC showed the highest concentrations in the No. 6 diet (13.33, 23.53 and 24.97 ng/g, respectively). The concentrations of HCB, β -BHC and γ -BHC in the No. 5 diet (8.15, 14.18 and 10.13 ng/g, respectively) were lower than those of the No. 6 diet. In addition, No. 5 diet was found to contain endrin (28.15 ng/g). HCB, γ -BHC and *t*-nonachlor were detected in the No. 2 diet (5.11, 8.3 and 14.28 ng/g, respectively) and the No. 1 and 4 diets were found to contain only *t*-nonachlor and HCB, respectively (8.41 and 1.62 ng/g, respectively). In the No. 3 diet, no organochlorine pesticides were detected.

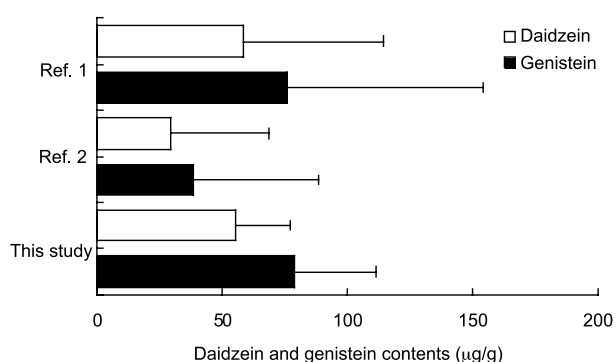


Fig. 1. Comparison of Phytoestrogen Levels in Fish Diets from Present and Previous Investigations

Data represented are the means and standard deviation. Ref. 1: Miyahara *et al.*, 2003 ($n = 8$); Ref. 2: Inudo *et al.*, 2004 ($n = 6$).

Estrogenic Activity for ER α and ER β

We evaluated the estrogenic activity of the fish diet for ER α and ER β using the yeast two-hybrid assay. No estrogenic activity was detected by the yeast two-hybrid assay for ER α and ER β without prior hydrolysis of the diets (data not shown). However, after hydrolysis by β -glucuronidase, the No. 2 and/or No. 3 diets showed the highest estrogenic activity for ER α and ER β . The EC $_{x10}$ values of the No. 2 diet for ER α and ER β were estimated to be 338.2 ± 46.8 and 75.2 ± 9.3 μ g/ml, respectively, and the E2 equivalents were noted as 84.2 ± 3.6 and 489.0 ± 16.6 ng/g, respectively (Table 1). Similarly, the EC $_{x10}$ values of the No. 3 diet for ER α and ER β were estimated to be 354.7 ± 1.6 and 70.1 ± 9.6 μ g/ml, respectively, and the E2 equivalents were noted at 72.8 ± 4.5 and 525.0 ± 8.1 ng/g, respectively (Table 1). The EC $_{x10}$ values of the No. 4 diet for ER β were estimated to be 253.8 ± 49.7 μ g/ml, and the E2 equivalent concentration was noted as 150.7 ± 8.5 ng/g (Table 1). The E2 equivalents of No. 1, 5 and 6 diets for ER α were estimated to be 54.0 ± 3.1 , 73.8 ± 3.5 and 27.7 ± 1.6 ng/g, respectively, and the E2 equivalents of these diets for ER β were calculated as 421.2 ± 26.3 , 494.0 ± 13.2 and 272.6 ± 6.1 ng/g, respectively (Table 1). However, in the No. 4 diet, the values of EC $_{x10}$ and E2 equivalents could not be determined as a result of the low estrogenic activity for ER α . All of the diets exhibited higher levels of activation of ER β than with ER α (Fig. 2).

Relationship between Estrogenic Activity and Phytoestrogens in the Fish Diet

In most diets (for ER α and ER β), a positive correlation was found between the E2 conversion from the phytoestrogen content and the estrogenic activity of E2 conversion by the yeast two-hybrid assay

Table 1. Levels of Phytoestrogens (Genistein and Daidzein) and Estrogenic Activities for ER α and ER β in Fish Diets

Diet No.	Phytoestrogen ^{a)}		Estrogenic activity ^{b)}			
	Genistein (μ g/g)	Daidzein (μ g/g)	ER α		ER β	
			EC $_{x10}$ (μ g/ml)	E2 equivalent (ng/g)	EC $_{x10}$ (μ g/ml)	E2 equivalent (ng/g)
1	79.9 ± 3.7	60.0 ± 2.1	445.2 ± 48.5	54.0 ± 3.1	92.6 ± 25.4	421.2 ± 26.3
2	115.7 ± 2.0	78.4 ± 1.1	338.2 ± 46.8	84.2 ± 3.6	75.2 ± 9.3	489.0 ± 16.6
3	118.9 ± 1.2	81.3 ± 0.8	354.7 ± 1.6	72.8 ± 4.5	70.1 ± 9.6	525.0 ± 8.1
4	39.7 ± 0.5	28.7 ± 0.3	$> 2500^c)$	$< 11.0^c)$	253.8 ± 49.7	150.7 ± 8.5
5	59.2 ± 1.9	40.1 ± 0.9	354.7 ± 18.5	73.8 ± 3.5	70.8 ± 6.5	494.0 ± 13.2
6	58.2 ± 0.5	42.1 ± 0.8	938.1 ± 85.0	27.7 ± 1.6	128.1 ± 8.2	272.6 ± 6.1

a) Data of phytoestrogen content represent the mean and standard deviation ($n = 3$). b) Data of estrogenic activity for ER α and ER β represent the mean and standard deviation ($n = 3$). c) Not detectable in the tested concentration range (39–2500 μ g fish diet/ml).

($p < 0.001$, Fig. 3).

DISCUSSION

Phytoestrogens and organochlorine pesticides in the diet used for laboratory test animals are a possible source of interference with bioassays that assess estrogenic activity. In the present study, we as-

sessed the levels of dietary phytoestrogens, organochlorine pesticides and the estrogenic activity of various diets for an experimental fish and discuss the potential contribution of these substances to estrogenic activity in comparison with those tested in previous studies.

Genistein (concentrations ranging from 39.7 to 118.9 $\mu\text{g/g}$) and daidzein (concentrations ranging from 28.7 to 81.3 $\mu\text{g/g}$) were detected in all fish diets tested in this study. These concentrations of phytoestrogens are consistent with our previous studies, and there were no differences between the regions tested in this study, namely Japan,²³⁾ the United Kingdom²²⁾ and Korea. Court and Freeman measured the genistein and daidzein content in different types of commercial cat food, and reported that genistein and daidzein were detected in 24 out of 42 types that were surveyed, within a concentration range of 1 to 163 $\mu\text{g/g}$.²⁴⁾ In this study, the No. 3 diet was found to contain the highest levels of genistein ($118.9 \pm 1.2 \mu\text{g/g}$) and daidzein ($81.3 \pm 0.8 \mu\text{g/g}$), but showed lower levels of genistein and daidzein than those found in commercial cat food. These results indicate that most types of commercial fish diet contain phytoestrogens, such as genistein and daidzein, as well as in the commercial cat food reported in a previous study.

In soybean, the following 12 isoflavone types have been identified: 3 types of aglycon (daidzein, genistein and glycitein), their glycosides (daidzin, genistin and glycitin), and their malonylized glycosides and acetylated glycosides.²⁵⁾ Among these isoflavones, the estrogenic activities of genistein and daidzein are reported to be higher than those of genistin and daidzin.²³⁾ In this study, we hydrolyzed the genistin and daidzin glycosides in the diet in order to measure the total amount of genistein and daidzein. The amount of glycosides without hydrolysis and the content of genistin and daidzin are unknown. However, no estrogenic activities were de-

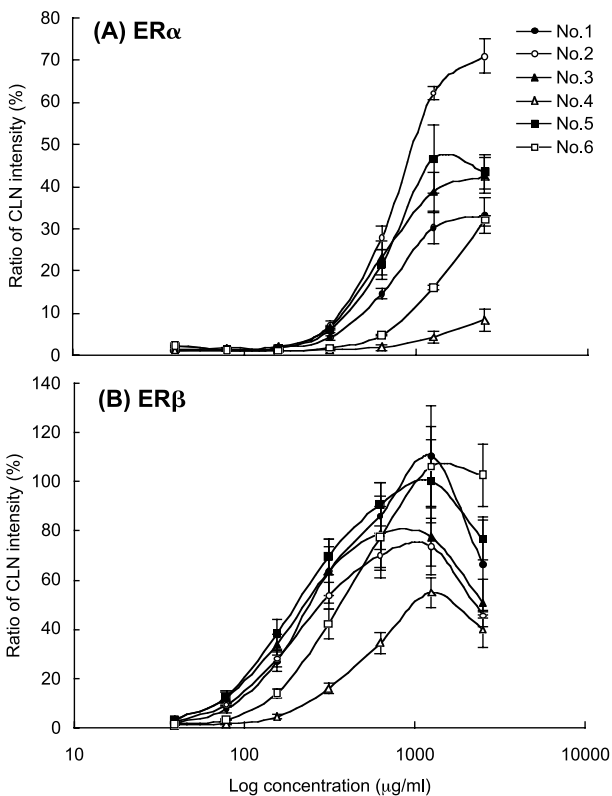


Fig. 2. Dose-Response Curves for Fish Diets after Hydrolysis Using the Agonist Test for the Yeast Two-Hybrid Assay for ER α (A) and ER β (B)

The values are calculated as a ratio of the chemiluminescence (CLN) intensity (T/B) of β -galactosidase. Data represented are the means and standard deviation ($n = 3$).

Table 2. Contents of Organochlorine Pesticides in Fish Diets using GC/ECD Analysis

Diet No.	HCB (ng/g)	β -BHC (ng/g)	γ -BHC (ng/g)	Vinclozolin (ng/g)	<i>p,p'</i> -DDE (ng/g)	<i>t</i> -nonachlor (ng/g)	Endrin (ng/g)	<i>o,p'</i> -DDT (ng/g)
1	N.D. ^{a)}	N.D. ^{b)}	N.D. ^{c)}	N.D. ^{d)}	N.D. ^{d)}	8.41	N.D. ^{f)}	N.D. ^{g)}
2	5.11	N.D. ^{b)}	8.3	N.D. ^{d)}	N.D. ^{d)}	14.28	N.D. ^{f)}	N.D. ^{g)}
3	N.D. ^{a)}	N.D. ^{b)}	N.D. ^{c)}	N.D. ^{d)}	N.D. ^{d)}	N.D. ^{e)}	N.D. ^{f)}	N.D. ^{g)}
4	1.62	N.D. ^{b)}	N.D. ^{c)}	N.D. ^{d)}	N.D. ^{d)}	N.D. ^{e)}	N.D. ^{f)}	N.D. ^{g)}
5	8.15	14.18	10.13	N.D. ^{d)}	N.D. ^{d)}	N.D. ^{e)}	28.15	N.D. ^{g)}
6	13.33	23.53	24.97	N.D. ^{d)}	N.D. ^{d)}	N.D. ^{e)}	N.D. ^{f)}	N.D. ^{g)}

N.D.: Quantity limit. a) < 0.05 ng/g; b) < 0.13 ng/g; c) < 0.1 ng/g; d) < 1.67 ng/g; e) < 0.5 ng/g; f) 1.0 ng/g; g) 0.83 ng/g.

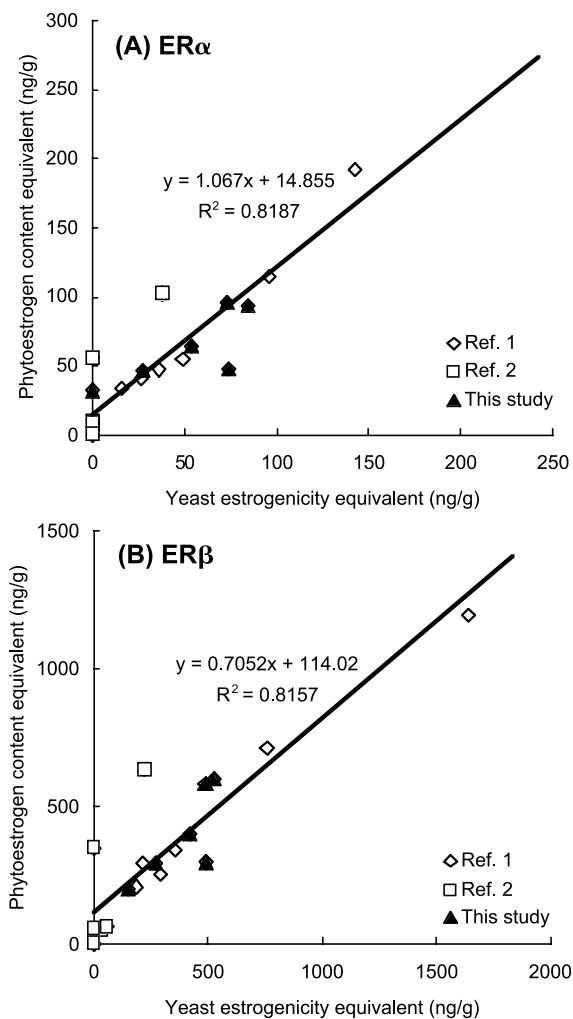


Fig. 3. Relationship between the Estrogenicity Equivalents from the Estrogenic Activities for ER α (A) and ER β (B) Using the Yeast Two-Hybrid Assay and the Estrogenicity Equivalents from Genistein and Daidzein Contents after HPLC Analysis of an Experimental Animal Diet

Ref. 1: Miyahara *et al.*, 2003 ($n = 8$); Ref. 2: Inudo *et al.*, 2004 ($n = 6$).

tected in the yeast two-hybrid assay for ER α and ER β without hydrolysis, although estrogenic activity was detected after hydrolysis. 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. Furthermore, all diets that had estrogenic activity showed higher affinity for the β receptor than for the α receptor. Kuiper *et al.*²⁶⁾ reported the estrogenic activity of genistein for human ER α and ER β , and the binding affinities of genistein for human ER α and ER β were 4 and 87, respectively, whilst that for E2 was set to 100. These results indicate that phytoestrogens, such as genistein and daidzein, were the main substances

contributing to the estrogenic activity of the diet.

Organochlorine pesticides, such as HCB, β -BHC, γ -BHC, *t*-nonachlor, and/or endrin were detected in most fish diets. Among these organochlorine pesticides, β -BHC and endrin have been reported as estrogenic pesticides in the reporter gene assay, and the 20% relative effective concentration (REC₂₀) values of β -BHC and endrin for human ER α were 3.5×10^{-7} and 1.8×10^{-6} M, respectively.²¹⁾ Considering the effective concentrations for human ER α , organochlorine pesticides such as β -BHC and endrin were not the main substances contributing to the estrogenic activity of the diet. However, it should be considered that the yeast two-hybrid system tested in this study basically shows lower responsiveness to estrogenic substances than that of the cultured animal cells.²⁷⁾ Moreover, live foods, such as *Artemia nauplii*, can be a source of adventitious xenobiotics. The different geographic strains of *Artemia* cysts may contain various amounts and types of chlorinated and aromatic hydrocarbons and heavy metals.²⁰⁾ Although adventitious contaminants such as organochlorine pesticides in unpurified commercial fish diets have not been evaluated in previous studies, our findings suggest that there are detectable levels of organochlorine pesticides in experimental fish diets.

To study the relationship between estrogenic activity and the phytoestrogen content of various diets from present and previous investigations, we evaluated the estrogenic activity of the feeding diet for experimental fish found by the yeast two-hybrid assay and the total amount of genistein and daidzein analysed by HPLC. In most diets (for ER α and ER β), a positive correlation was found between the E2 conversion from the phytoestrogen content (Fig. 3-Y axis) and the estrogenic activity of E2 conversion by the yeast two-hybrid assay (Fig. 3-X axis). Therefore, it is strongly suggested that genistein and daidzein, which are phytoestrogens, largely contribute to the estrogenic activity of these diets for ER α and ER β . However, some diets, such as Nos. 2, 3 and 5, were outside the regression curve in the relationship between the E2 conversion of the phytoestrogen content in the diet and the estrogenic activity for E2 conversion by the yeast two-hybrid assay. In the No. 5 diet, HCB, β -BHC, γ -BHC and endrin were detected at 8.15, 14.18, 10.13 and 28.15 ng/g, respectively. It is unclear whether there are combined effects of these substances with estrogenic activities from the fish diet. Furthermore, organochlorine pesticides, such as β -BHC and endrin

have been reported as estrogenic substances,²¹⁾ although these organochlorine pesticides were not the main substances contributing to the estrogenic activity of the diet. Kojima *et al.* revealed anti-androgenic activity of pesticides such as vinclozolin and *p,p'*-DDE.²¹⁾ The plants used as raw materials for the fish diet may contain other chemicals with anti-estrogenic and (anti-)androgenic effects, although vinclozolin and *p,p'*-DDE were not detected in the fish diets tested in this study. The effects of (anti-)estrogenic and (anti-)androgenic substances in the fish diet should also be considered.

Our previous study reported that phytoestrogens (genistein, 58.5 µg/g; daidzein, 37.3 µg/g) were detected in a commercial fish diet, and this diet induced hepatic Vg synthesis in male medaka (*Oryzias latipes*).²²⁾ Moreover, we reported that plasma Vg was detected in male goldfish fed 1.0% body weight of the diet containing phytoestrogens (genistein, 47.7 µg/g; daidzein, 41.1 µg/g) every two days for 31 days.²⁸⁾ In the present study, genistein (concentrations ranging from 39.7 to 118.9 µg/g) and daidzein (concentrations ranging from 28.7 to 81.3 µg/g) were detected in all fish diets. These dietary concentrations of phytoestrogens with the exception of the No. 4 diet could be sufficiently high to cause the production of plasma Vg in male medaka and goldfish. Furthermore, our previous study¹⁸⁾ reported the effects of the xenoestrogen nonylphenol on the production of plasma Vg with the measurement of steroid hormones in goldfish fed on a diet with a high phytoestrogen content. The conclusion was that a large amount of phytoestrogens in the fish diet competed with nonylphenol in binding ER in the *in vivo* Vg production assay and may affect the estimation of the estrogenic activity of nonylphenol. These results strongly suggest that it is necessary to assess the estrogenic activity or contents of phytoestrogens such as genistein and daidzein in the fish diet when performing an *in vivo* screening assay for EDCs. Therefore, we recommend the use of a standardized, open-formula diet in which estrogenic substances have been reduced to levels that do not alter the results of studies which are influenced by exogenous estrogens.

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