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Removal of estrogenic activity of endocrine-disrupting genistein by ligninolytic enzymes from white rot fungi

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Abstract

Endocrine-disrupting genistein was treated with the white rot fungus *Phanerochaete sordida* YK-624 under ligninolytic condition with low-nitrogen and high-carbon culture medium. Genistein decreased by 93% after 4 days of treatment and the activities of ligninolytic enzymes, manganese peroxidase (MnP) and laccase, were detected during treatment, thus suggesting that the disappearance of genistein is related to ligninolytic enzymes produced extracellularly by white rot fungi. Therefore, genistein was treated with MnP, laccase, and the laccase-mediator system with 1-hydroxybenzotriazole (HBT) as a mediator. HPLC analysis demonstrated that genistein disappeared almost completely in the reaction mixture after 4 h of treatment with either MnP, laccase, or the laccase-HBT system. Using the yeast two-hybrid assay system, it was also confirmed that three enzymatic treatments completely removed the estrogenic activity of genistein after 4 h. These results strongly suggest that ligninolytic enzymes are effective in removing the estrogenic activity of genistein.

Keywords: Genistein; Estrogenic activity; Ligninolytic enzyme; Manganese peroxidase; Laccase; White rot fungi

1. Introduction

Exposure to bleached kraft pulp mill effluent (BKME) has been associated with a multitude of reproductive responses in fish. Specifically, fish exposed to BKME have lower circulating concentrations of reproductive hormones, reduced gonad size, increased age of sexual maturation, smaller eggs, reduced expression of secondary sex characteristics, and elevated hepatic mixed-function oxygenase activity when compared with fish from reference sites [1-3]. Several studies have shown that these responses may be induced in fish through exposure to phytochemicals originating from wood components, including alkyl-substituted polynuclear aromatic hydrocarbons [4], phytosterols [5-7] and stilbenes [8]. Moreover, recent study has indicated that the isoflavonoid genistein may contribute to the changes in sex steroid levels and decreased reproductive capacity observed in fish near wood pulp mills. For example, genistein was quantified at concentrations of 13.1 μg/L and 10.5 μg/L in untreated and treated (final) BKME, respectively, from a wood pulp mill in Ontario, Canada [9]. This result indicates that genistein persists to a large degree through the wastewater treatment process. Furthermore, another report found that genistein was present at concentrations ranging from 2.7 to 38 ng/L in 60% of the effluents from 18 sewage treatment plants in Germany [10].

Genistein is a known endocrine disruptor substance that binds to the estrogen receptor and mimics the action of endogeneous sex steroids [11, 12]. Recently,

Kiparissis et al. demonstrated that there is a significant concentration-dependent increase in the numbers of male Japanese medaka (*Oryzias latipes*) with connective tissue in the testis and fibrosis around the testicular lobules, as well as an increase in the numbers of male fish showing low densities of spermatozoa in both the testicular lobules and efferent ducts [13]. This study indicates that genistein should be considered a candidate estrogenic compound that may be involved in the alteration of sexual development in feral fish populations.

Great interest is currently being expressed in lignin-degrading white rot fungi and their ligninolytic enzymes due to a recognized potential for degrading and detoxifying recalcitrant environmental pollutants such as dioxins [14], chlorophenols [15], polycyclic aromatic hydrocarbons [16, 17], and dyes [18, 19]. Manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase produced extracellularly by lignin-degrading fungi have been demonstrated to be involved in the degradation of lignin and these pollutants.

MnP is a heme peroxidase produced by white rot fungi and catalyzes the oxidation of Mn(II) to Mn(III) in the presence of hydrogen peroxide (H_2O_2). Malonate, oxalate, and α-hydroxy acids such as malate, lactate, and tartrate chelate the generated Mn(III) and release Mn(III) from the manganese-binding site of MnP. The released Mn(III)-organic acid complex in turn oxidizes various phenolic compounds, including lignin [20, 21]. Fungal laccase is a multicopper oxidase that catalyzes single-electron

oxidation of phenolic compounds by reducing molecular oxygen to water [22]. In the presence of a mediator such as 1-hydroxybenzotriazole (HBT) or 2,2´-azinobis (3-ethylbenzothiazoline-6-sulfonate), laccase is capable of oxidizing nonphenolic compounds that it cannot oxidize alone [23, 24]. We recently demonstrated that MnP, laccase, and the laccase-HBT system are effective in removing the estrogenic activities of bisphenol A (BPA), nonylphenol (NP), 17β-estradiol (E_2), and ethinylestradiol (EE_2) [25, 26] and in degrading methoxychlor [27] and a antifouling compound Irgarol 1051 [28]. These studies encouraged us to investigate the removal of estrogenic activity of genistein by ligninolytic enzymes, which are able to degrade various aromatic compounds.

In this study, we examined the disappearance of genistein by white rot fungus *Phanerochaete sordida* YK-624 and investigated the enzymes related to its disappearance. Furthermore, we applied related enzymes, MnP and laccase, to the treatment of genistein and describe the removal of its estrogenic activity as measured by a yeast two-hybrid system [29].

2. Materials and methods

2.1 Treatment of genistein with white rot fungus

P. sordida YK624 (ATCC 90872) was used in this study. The fungus was maintained in potato dextrose agar (PDA; Difco Laboratories) slants. A new PDA plate

was inoculated with the fungus, which was then precultured for 5 days at 30ºC. Five disks punched from the grown edge of the precultured mycelium on the PDA plate were homogenized for 30 s with 50 ml of PMY medium (3.0% glucose, 1.0% peptone, 1.0% malt extract, 0.4% yeast extract), which was then put into a 500-ml Erlenmeyer flask with 150 ml of PMY medium and shaken at 150 rpm and 30ºC to give a mycelium suspension culture. After 3 days, 2.2 ml of the culture was homogenized once again for 30 s with 20 ml of Kirk medium [30] and then added to a 100-ml Erlenmeyer flask containing 10^{-4} M genistein (Fig. 1, Wako, Osaka, Japan). The flask was shaken at 150 rpm and 30ºC.

2.2 Enzyme assay and preparation

Laccase activity was determined by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) at 470 nm and 30ºC. The reaction mixture contained 1 mM DMP and 50 mM malonate buffer (pH 4.5). MnP activity was determined in the same manner except that the reaction mixture also contained 0.1 mM $MnSO_4$ and 0.2 mM H_2O_2. LiP activity was determined by monitoring the oxidation of veratryl alcohol (VA) at 310 nm and 30ºC. The reaction mixture contained 1 mM VA, 20 mM succinate buffer (pH 3.0), and 0.2 mM H_2O_2. One katal (kat) of enzyme activity is the amount of enzyme producing 1 mol of the quinone dimer (49.3 $mM^{-1}cm^{-1}$) or veratraldehyde (9.3 $mM^{-1}cm^{-1}$) from DMP or VA, respectively, per second [31, 32].

Partially purified MnP and laccase were prepared from cultures of *P. sordida* YK-624 and *Trametes versicolor* IFO-6482, respectively, as described in previous reports [25, 33]. Each partially purified ligninolytic enzyme preparation contained no other ligninolytic enzyme activities.

2.3 Treatment of genistein with ligninolytic enzymes

For treatment with MnP, the reaction mixture consisted of 10^{-4} M genistein, partially purified MnP (10 nkat/ml), 50 mM malonate buffer (pH 4.5), $MnSO_4$ (0.1 mM), Tween 80 (0.1%), and glucose (25 mM) and glucose oxidase (3.33 nkat/ml; Wako, Osaka, Japan) to supply H_2O_2. The reaction was performed at 30ºC while stirring at 150 rpm. Laccase treatment was conducted in the same manner except that laccase (10 nkat/ml) was used instead of MnP, and $MnSO_4$, Tween 80, glucose, and glucose oxidase were omitted from the reaction mixture. For the laccase-mediator system, 0.2 mM 1-hydroxybenzotriazole (HBT) was added to the reaction mixture for laccase treatment [34].

2.4 Analyses of genistein treated with white rot fungus or ligninolytic enzymes

Residual genistein concentrations in the fungal or enzymatic reaction mixtures were determined by high-performance liquid chromatography (HPLC) analysis. HPLC analyses were carried out with a Wakosil-II 5C18HG (Wako) column using an isocratic

elution with 37% acetonitrile aqueous containing 1% acetic acid at a flow rate of 1 ml/min with detection at 260 nm.

2.5 Estrogenic activity of genistein treated with ligninolytic enzymes

The estrogenic activity of genistein before and after enzymatic treatment was evaluated by an *in vitro* screening test for chemicals with hormonal activities that used the yeast two-hybrid estrogenic assay system, developed by Nishikawa *et al.* [29]. The transformed yeast Y190 with the pGBT9-estrogen receptor ligand-binding domain (pGBT9-ER LBD) and pGAD424-coactivator was provided by Nishihara, Osaka University. In the yeast GAL4 DNA binding domain-ER LBD and GAL4 activation domain-coactivator fusion proteins were expressed from these expression plasmid. The Y190 harbors a GAL4 binding site upstream of a *lacZ* gene, so that β-galactosidase activity corresponds to the strength of interaction between estrogen receptor and coactivator. The protein-protein interaction between estrogen receptor and coactivator was strictly dependent on the presence of estrogen [29].

In the assay system, when 2.5 μl of reaction mixture containing 10^{-4} M genistein is added to 50 μl of yeast culture and 200 μl of SD medium [25], the concentration of genistein before enzymatic treatment is 10^{-6} M. As shown in Fig. 4, the estrogenic activity of genistein is low at this concentration (10^{-6} M) in the assay system. Therefore, the test sample (2.5 μl) for the assay system was prepared as

follows; each reaction mixture (25 μl) was lyophilized and dissolved in dimethyl sulfoxide (2.5 μl), which means that the concentration of genistein before enzymatic treatment corresponds to 10^{-5} M in the assay system. Relative estrogenic activity (%) was defined as the percentage of β-galactosidase activity of enzyme-treated genistein compared to that of untreated genistein. For the experiments described in Fig. 4, 2.5 μl of various concentrations of BPA, NP, and E_2 dissolved in dimethyl sulfoxide were used to evaluate their estrogenic activities instead of reaction mixtures.

3. Results and Discussion

3.1 Treatment of genistein with white rot fungus

Ligninolytic activity of white rot fungi appears as a secondary metabolic event, and nutritional nitrogen or carbon limitation (or both) allows extensive degradation of lignin [35-37]. Thus, the treatment of genistein with *P. sordida* YK-624 was investigated under ligninolytic condition with low-nitrogen and high-carbon culture medium. Figure 2 shows the decrease in genistein observed during treatment with *P. sordida* YK-624. No appreciable decrease in genistein was observed during the first two days of treatment, but marked decrease was noted after 2 days of treatment and the concentration of genistein decreased by 93% after 4 days of treatment.

It has been suggested that MnP, LiP, and laccase produced extracellularly by white rot fungi are involved in the oxidative breakdown of lignin [38-44]. Therefore,

these enzyme activities were determined during treatment of genistein with *P. sordida* YK-624 (Fig. 2). MnP and laccase activities were detected after 2 days of treatment, but LiP activity was not detected throughout the treatment. The profiles of MnP and laccase productions and genistein decrease during treatment were very similar. These results strongly suggest that MnP and laccase are involved in the disappearance of genistein by *P. sordida* YK-642.

3.2 Treatment of genistein with ligninolytic enzymes

We applied three enzymatic treatments, MnP, laccase, and the laccase-HBT system, to remove the estrogenic activity of genistein. As shown in Fig. 3, HPLC detection with an ODS column (260 nm) of the residual genistein during these three enzymatic treatments revealed that genistein decreased by over 90% after 1 h of treatment, and almost completely disappeared in the reaction mixture after 4 h of treatment with either MnP, laccase, or the laccase-HBT system. This indicates that all three enzymatic treatments are effective in the consumption of genistein. However, the greatest focus concerning the biodegradation of an endocrine-disrupting (estrogenic) chemical should be on the removal of this activity. We therefore attempted to assay the estrogenic activity of the reaction mixtures of genistein during enzymatic treatment using the yeast two-hybrid estrogenic assay system. This system is newly developed and is based on the ligand-dependent interaction between the nuclear hormone receptor

and its coactivator. The method is rapid and has been confirmed to be reliable for measuring estrogenic activity [29].

The estrogenic activity of genistein, expressed as β-galactosidase activity, is compared with those of BPA, NP, and E_2, which are known endocrine disruptors (Fig. 4). The activity of genistein (10^{-5} M) was almost the same as those of BPA (10^{-4} M), NP (10^{-5} M), and E_2 (~5×10^{-9} M). This finding is consistent with a previous report in which the relative estrogenicities of genistein, BPA, and NP were 4×10^{-4}, 5×10^{-5}, and 4×10^{-4}, respectively, as compared with E_2 [45].

Figure 5 shows that MnP, laccase, and the laccase-HBT system reduced the estrogenic activity of genistein by over 90% after 1 h of treatment and completely removed the activity after 4 h of treatment. This was consistent with the HPLC data on the disappearance of genistein shown in Fig. 3. In HPLC analyses, we detected traces of unknown compounds, presumably degradation products, in the reaction mixtures after 1 h and 4 h of treatment with either MnP, laccase, or the laccase-HBT system. However, their peak area was much less than the amount of genistein consumed, even if their absorption coefficients were different. In a previous report, we demonstrated that treatment of BPA and NP with MnP, laccase, and the laccase-HBT system resulted in oligomeric reaction products through the formation of phenoxy radicals of these chemicals followed by radical coupling [25]. Thus, the removal of estrogenic activity of genistein may be due to polymerization brought about by enzymatic oxidation,

because genistein has a *para*-substituted phenol structure, as do BPA and NP.

Very little is known about the disappearance of genistein, and no data exist on the microbial removal of the estrogenic activity of genistein. Therefore, this is the first report that ligninolytic enzymes MnP and laccase from white rot fungus can effectively decrease genistein and remove its estrogenic activity.

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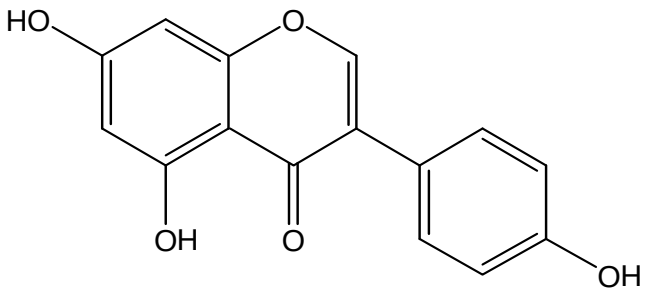

Fig. 1 Chemical structure of genistein.

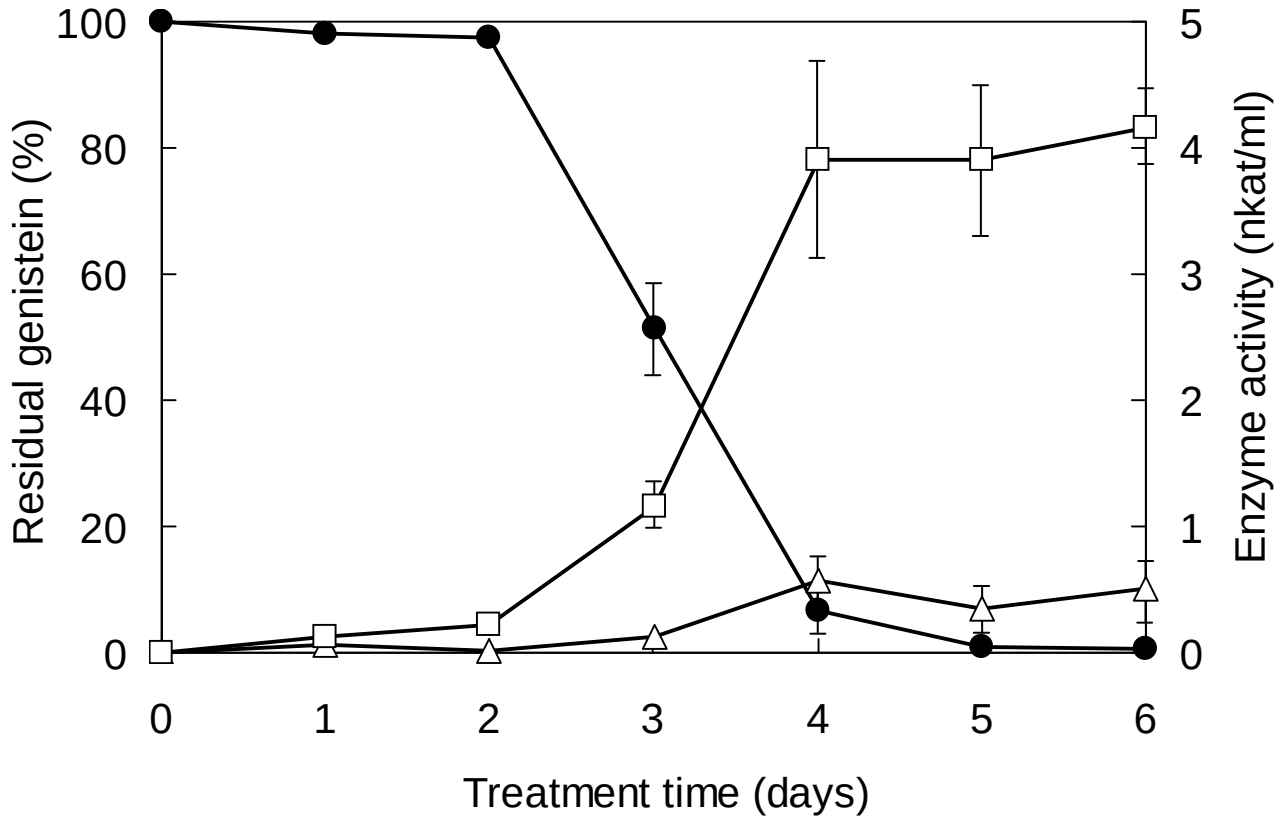

Fig. 2 Treatment of genistein with *P. sordida* YK-624. Indicated for each point are the mean and standard deviation of triplicate cultures. Filled circles, Residual genistein; open squares, MnP activity; open triangles, laccase activity.

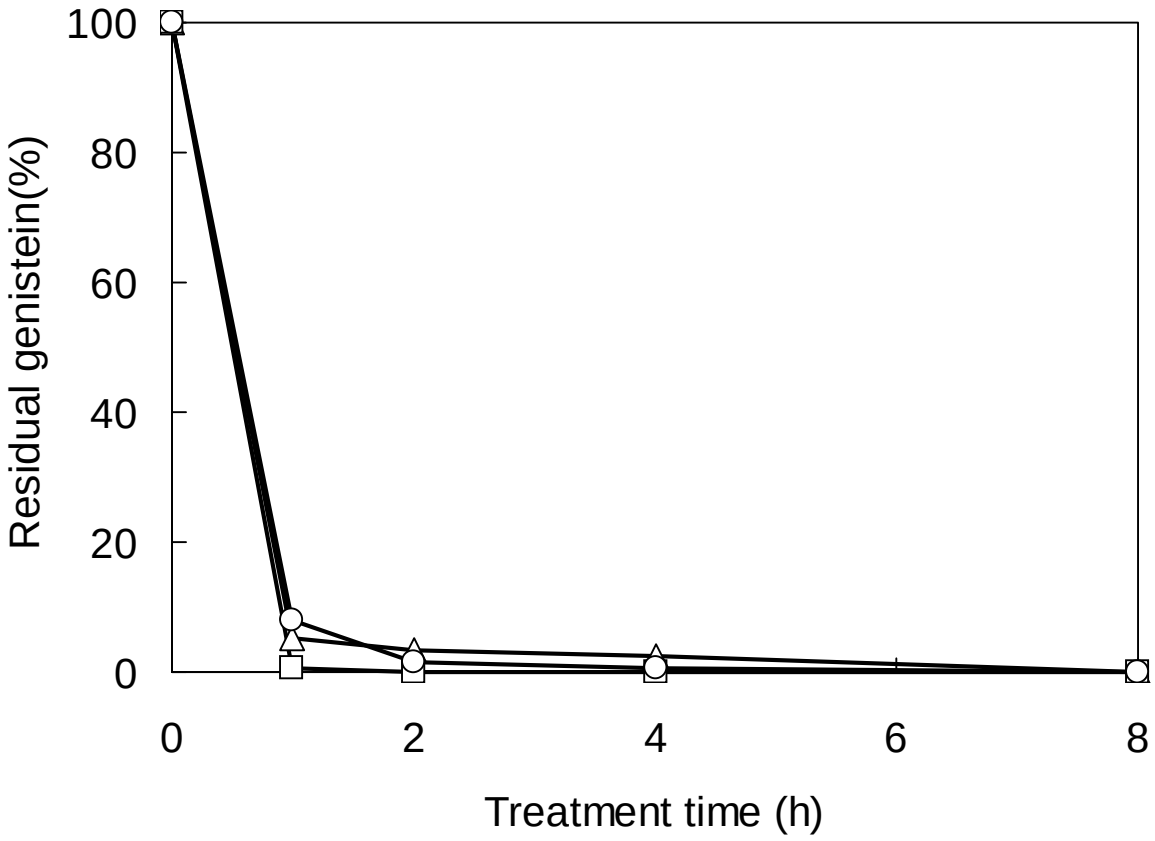

Fig. 3 Decrease in genistein concentration by enzymatic treatment with MnP, laccase, or laccase-HBT system. Open circles, MnP; open triangles, laccase; open squares, laccase-HBT system.

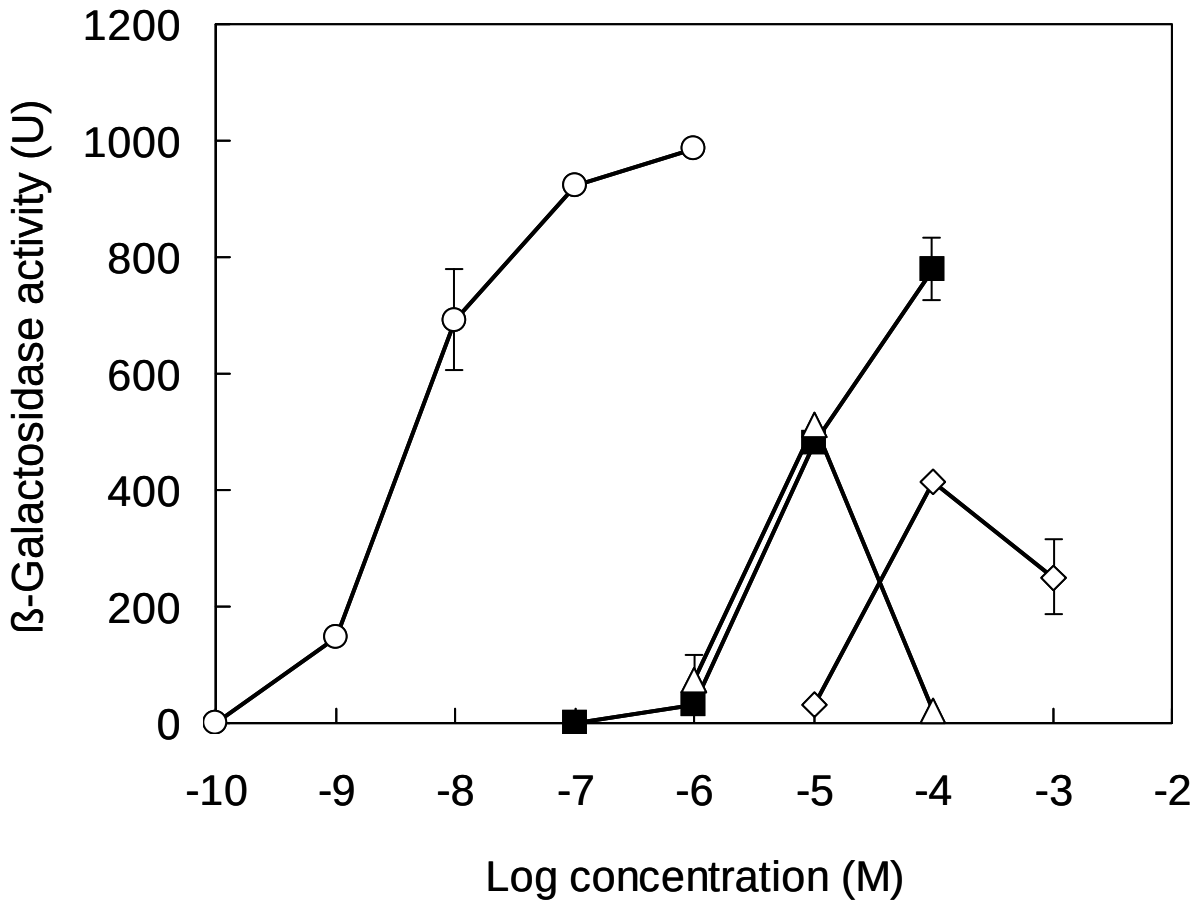

Fig. 4 Dose response curve for estrogenic activity as measured by yeast two-hybrid assay. Indicated for each point are the mean and standard deviation of five experiments for genistein, BPA, NP, and E_2. Filled squares, genistein; open diamonds, BPA; open triangles, NP; open circles, E_2.

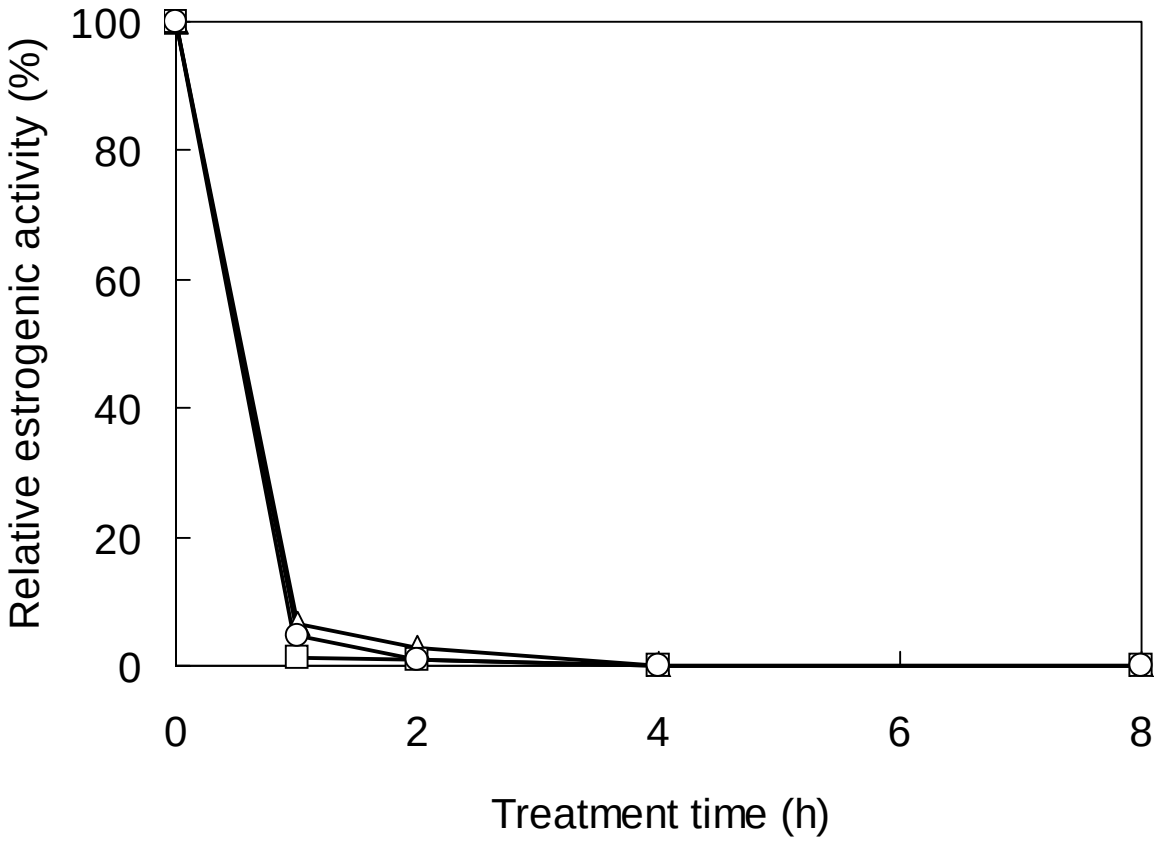

Fig. 5 Removal of estrogenic activity of genistein by enzymatic treatment with MnP laccase, or laccase-HBT system. Open circles, MnP; open triangles, laccase; open squares, laccase-HBT system.