

Removal of estrogenic activities of bisphenol A and nonylphenol by oxidative enzymes from lignin-degrading basidiomycetes

Yuji Tsutsumi, Takashi Haneda, Tomoaki Nishida *

Department of Forest Resources Science, Shizuoka University, Ohya 836, Shizuoka 422-8529, Japan

Corresponding author: ***T. Nishida**

Tel and Fax: +81-54-238-4852

E-mail: aftnisi@agr.shizuoka.ac.jp

Abstract

Bisphenol A (BPA) and nonylphenol (NP) were treated with manganese peroxidase (MnP) and laccase prepared from the culture of lignin-degrading fungi. Laccase in the presence of 1-hydroxybenzotriazole(HBT), the so-called laccase-mediator system, was also applied to remove the estrogenic activity. Both chemicals disappeared in the reaction mixture within a 1-h treatment with MnP but the estrogenic activities of BPA and NP still remained 40% and 60% in the reaction mixtures after a 1-h and a 3-h treatment, respectively. Extension of the treatment time to 12 h completed the removal of estrogenic activities of BPA and NP. The laccase has less ability to remove these activities than MnP, but the laccase-HBT system was able to remove the activities in 6 h. A gel permeation chromatography (GPC) analysis revealed that main reaction products of BPA and NP may be oligomers formed by the action of enzymes. Enzymatic treatments extended to 48 h did not regenerate the estrogenic activities, suggesting that the ligninolytic enzymes are effective for the removal of the estrogenic activities of BPA and NP.

Keywords: Manganese peroxidase ; Laccase ; Laccase-mediator ; Bisphenol A ; Nonylphenol ; Estrogenic activity ; Fungi

1. Introduction

Considerable concern has recently been expressed over the possibility that some man-made chemicals that mimic the effects of hormones (particularly estrogens) may adversely affect reproduction in wildlife and humans (Colborn and Clement, 1992; Stone, 1994). Like natural estrogens, these estrogenic chemicals can bind to the estrogen receptor and regulate the activity of estrogen responsive genes. Therefore, such effects have raised concern that prolonged exposure to environmentally relevant concentrations of these chemicals could result in reproductive toxicity. Bisphenol A (2, 2-bis(4-hydroxyphenyl)propane; BPA) and nonylphenol (NP) are widely used in a variety of industrial and residential applications, and are suspected of having estrogenic (endocrine-disrupting) activity (Sato et al., 1991; While et al., 1994; Perez et al., 1998; Schaer et al., 1999). It was reported that a gram-negative aerobic bacterium can degrade BPA (Lobos et al., 1992; Spivack et al., 1994) and that an aerobic bacterium, tentatively identified as a *Sphingomonas* sp., utilizes NP as its only carbon and energy source (Tanghe et al., 1999). However, degradation of BPA and NP by microorganisms other than bacteria has not yet been attempted. There is currently great interest in the lignin-degrading fungi and ligninolytic enzymes because their industrial potentials are recognized in biomechanical pulping (Kashino et al., 1993), biobleaching (Paice et al., 1993; Katagiri et al., 1995; Ehara et al., 1997), and degradation and detoxification of recalcitrant environmental pollutants such as dioxins (Bumpus et al., 1985),

chlorophenols (Joshi and Gold, 1993), nylon (Deguchi et al., 1997; Deguchi et al., 1998), polyethylene (Iiyoshi et al., 1998; Ehara et al., 2000), and dyes (Ollikka et al., 1993; Nishida et al., 1999). It has been demonstrated that laccase, manganese peroxidase (MnP), and/or lignin peroxidase (LiP) produced extracellularly by lignin-degrading fungi are involved in the degradation of lignin and these pollutants. Both BPA and NP are phenolic compounds and they are therefore good substrates for the above ligninolytic enzymes as well as for lignin. In this study, we applied the ligninolytic enzymes, laccase and MnP, to the treatments of BPA and NP, and described the removal of their estrogenic activities assayed by the two-hybrid yeast system (Nishikawa et al., 1999).

2. Materials and methods

2.1. Chemicals and enzyme preparations

BPA and NP used in this study were extra pure grade purchased from Tokyo Chemical Industry (Tokyo, Japan). All other chemicals were also extra pure grade, obtained from commercial sources, and used without further purification. Partially purified MnP and laccase were prepared from the culture of *Phanerochaete chrysosporium* ME-446 and *Trametes versicolor* IFO70473, respectively, according to our previous reports (Katagiri et al., 1997; Nishida et al., 1999). The partially purified MnP did not contain any laccase, and vice versa. LiP activity was not detected in both

enzyme preparations. However, the number of isoenzymes in each enzyme was not confirmed.

2.2. Enzyme assay

Laccase activity was determined by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) at 470 nm. The reaction mixture contained 1 mM DMP, 50 mM malonate buffer (pH 4.5). MnP activity was determined in the same manner except that the reaction mixture contained 0.1 mM MnSO₄ and 0.2 mM H₂O₂. One unit of each enzyme activity is defined as the amount of enzyme producing 1 μmol of the quinone dimer per min from DMP (Wariishi et al., 1992).

2.3. Enzymatic treatments of BPA and NP

For the MnP treatment, the reaction mixture consisted of BPA (0.22 mM) or NP (0.23mM), partially purified MnP (100 mU/ml), 50 mM malonate buffer (pH 4.5), MnSO₄ (50 μM), and glucose (25 mM) and glucose oxidase (2 U; Wako, Osaka, Japan) to supply H₂O₂. In the case of NP, the above reaction mixture contained 0.5% methanol and 0.075% Tween 80 to facilitate the solubility of NP. The reaction was carried out at 30°C with stirring at 150 rpm. The laccase treatment was performed in the same manner except that laccase (100mU/ml) was used instead of MnP, and MnSO₄, glucose, and glucose oxidase were omitted from the reaction mixture. For the laccase-mediator

system, 0.2 mM 1-hydroxybenzotriazole (HBT) was added into the reaction mixture for laccase treatment (Bourbonnais et al., 1997).

2.4 .Analyses of BPA and NP treated with enzyme

Residual BPA in the enzymatic reaction mixture was determined by high-performance liquid chromatography (HPLC) analysis. The analytical conditions were as follows:

Column: Wakosil-II5C18HG (Wako);

Eluent: linear gradient from 0% to 95% of acetonitrile in water containing 0.1% trifluoroacetic acid;

flow rate: 0.6 ml/min;

detection: 275 nm.

HPLC analysis of NP was also performed using Wakosil-II5C8HG (Wako) column using an isocratic elution with 80% methanol at 0.8 ml/min, and detection at 277 nm.

The reaction mixture was subjected to gel permeation chromatography (GPC) to determine changes in molecular weight distribution of BPA and NP. For GPC analysis of BPA, a TSKgel G3000PW column (Tosoh, Tokyo), a mobile phase consisting of 0.05N NaOH, a flow rate of 0.6 ml/min, and detection at 275 nm were used. For GPC analysis of NP, a TSKgel α -M (Tosoh), a mobile phase consisting of 90% methanol and containing 12 mM lithium chloride, a flow rate of 0.8 ml/min, and detection at 277 nm

were used.

2.5. Estrogenic activities of BPA and NP treated with enzyme

The enzymatic treatment of BPA or NP was carried out according to the above method except that 0.88 mM BPA, 0.92 mM NP, and 800 mU/ml MnP or laccase were used. The estrogenic activity of BPA or NP was evaluated by an in vitro screening test for chemicals with hormonal activities using yeast, which has been developed by Nishikawa et al. (1999). 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 GAL4DNA binding domain-ERLBD and GAL4 activation domain-coactivator fusion proteins were expressed from these expression plasmids. 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 (Nishikawa et al., 1999).

The yeast was grown overnight at 30°C with shaking (150 rpm) in 1 ml of SD medium [26.7 g of a Minimal SD Base (Clontech Palo Alto, CA) per liter and 0.64 g of a –Leu/-Trp DO Supplement (Clontech) per liter]. The 50 μ l of overnight culture was then added to 200 μ l of fresh SD medium containing 2.5 μ l of reaction mixture of BPA or N treated with or without enzyme. After yeast cells were cultured for 4 h at 30°C in a

culture tube rotator (Type MBS-1B; EYELA, Tokyo, Japan) at 50 rpm, OD₆₀₀ of culture was determined. The yeasts were collected by centrifugation (15000 rpm, 5 min) from 100 µl of culture and resuspended in 200 µl of Z buffer [0.1 M sodium phosphate (pH 7.0), 10 mM KCl, 1 mM MgSO₄] containing 0.54 µl of β-mercaptoethanol and 0.2 mg of Zymolyase 20T (Seikagaku Corporation, Tokyo). Samples were incubated for 15 min at 30°C and then enzymatic reaction was started by addition of 40 µl of 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside. When a yellow color developed (30 min), 100 µl of 11N Na₂CO₃ was added to stop the reaction and OD₄₂₀ and OD₅₅₀ of supernatant after centrifugation (15000 rpm, 5 min) were determined. β-Galactosidase activity (U) was calculated by the following equation:

$$U = (OD_{420} - 1.75OD_{550}) \times 10^3 / tvOD_{600},$$

Where *t* is the time of reaction (30 min), *v* volume of culture used in assay (0.06 ml), OD₆₀₀ the cell density at the start of the assay, OD₄₂₀ the absorbance by *o*-nitrophenol at the end of the reaction and OD₅₅₀ is the light scattering at the end of the reaction. Relative estrogenic activity (%) is defined as the percentage of β-galactosidase activity of enzyme-treated BPA or NP compared to that of untreated BPA (421±46.6 U) and NP (613 ± 39.3 U).

3. Results and discussion

3.1. Disappearance of BPA and NP by treatments with ligninolytic enzymes

MnP is a heme peroxidase produced by white rot basidiomycetes fungi and expresses the oxidation of phenolic compounds in the presence of Mn(II) and H₂O₂. In the MnP catalyzing oxidation, chelate complexes of Mn(III) with organic acid such as malonate, lactate, or tartarate oxidize phenolic compounds, including lignin (Wariishi et al., 1992). A fungal laccase is a multicopper oxidase and catalyzes one-electron oxidation of phenolic compounds by reducing oxygen to water (Reinhammar, 1984). It has been demonstrated that the substrate range of fungal laccase is extended in the presence of a mediator such as HBT or 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate). The so-called laccase mediator system has been applied for the oxidative breakdown of lignin (Bourbonnais et al., 1997) and degradation of some environmental pollutants, e.g. phosphorothiolates (Amitai et al., 1998) and polycyclic aromatic hydrocarbons (Johannes et al., 1996; Pickard et al., 1999). Therefore, we applied three enzymatic treatments, MnP, laccase and laccase-HBT system, to remove the estrogenic activities of BPA and NP.

Both NP and BPA disappeared within a 1-h treatment with MnP. Laccase treatment also removed BPA by 70% and NP by 60% in an hour (Fig. 1(A) and (B)). Laccase has less ability to remove BPA and NP than MnP, but the addition of HBT in the laccase treatment greatly improved its potential. The reaction of BPA and NP with laccase

occurs via direct enzyme-substrate interaction, therefore, the reaction strongly depends on the substrate. On the other hand, Mn(III) chelate and HBT are involved in the oxidative reactions of MnP and laccase-HBT system, respectively. These systems thus can deal with a somewhat wider range of substrates than laccase. The half-lives of BPA were estimated to be 2.5-4 days in receiving waters (Dorn et al., 1987). 80-90% of BPA was converted into metabolites within a 3-h incubation by the resting-cell prepared from a gram-negative aerobic bacterium MV1 (Spivack et al., 1994). In our preliminary experiment, BPA and NP were treated with two of the best studied white rot fungi, *P. chrysosporium* and *T. versicolor*, and we confirmed that most of both chemicals disappeared in one week of culture (data not shown). Compared to the above reports, the ligninolytic enzyme-treatments thus achieved more rapid removal of BPA and NP.

3.2. Removal of the estrogenic activities of BPA and NP by treatments with ligninolytic enzymes

Although studies have been made on the biodegradation of BPA and NP, these studies have dealt with the consumption or disappearance of the targeted chemicals. The greatest concern for the biodegradation of an endocrine-disrupting chemical should be focussed on the removal of its activity. We therefore attempted the estrogenic assay of the reaction mixtures of BPA and NP during the enzymatic treatment using the two-hybrid estrogenic assay system in yeast. This system was newly developed by

Nishikawa et al. (1999) and is based on the ligand-dependent interaction between nuclear hormone receptor and coactivator. The method is rapid and has been confirmed to be reliable for measuring estrogenic activity (Nishikawa et al., 1999). The estrogenic activity of BPA remained at 40% and 20% in the reaction mixtures MnP and the laccase-HBT system, respectively (Fig. 2(A)). Also, in the case of NP with MnP and laccase treatment, 60% and 90% of its estrogenic activity remained after a 3-h treatment, respectively (Fig. 2(B)). In contrast, it was confirmed that no detectable level of residual BPA or NP remained in the reaction mixtures (data not shown). These results strongly indicate that the estrogenic assay is necessary in studies of the degradation of endocrine-disrupting chemicals. The estrogenic activities of BPA and NP were almost entirely removed by extension of the MnP and laccase treatments to 12 h (Fig. 2). The laccase-HBT system removed approximately 80% of the estrogenic activity of NP in just a 1-h treatment, and achieved almost complete removal of it within 6 h (Fig. 2(B)). It should also be noted that the estrogenic activities of BPA and NP never regenerate during any of the enzymatic treatments with MnP, laccase, or the laccase-HBT system, even if extended to 48 h (data not shown).

3.3. Fate of BPA and NP by the enzymatic treatment

In the determination of residual BPA and NP during the enzymatic treatment by HPLC equipped with an ODS column, we detected traces of unknown compounds,

presumably some degradation products, in the elution profiles of the reaction mixtures after a 12-h treatment. However, their peak area was much less than the amount of consumed BPA or NP, even if considering their absorption coefficients were different (data not shown). The GPC analysis of the reaction mixture revealed that the reaction products from BPA or NP were eluted faster than each chemical. It is reasonable that formation of phenoxy radicals of these chemicals followed by radical coupling would occur during the treatments with MnP, laccase, or the laccase-HBT system. The coupling reaction occurs and the reaction products must lose structural characteristics of acting as estrogens.

Fig. 3 shows typical GPC profiles of the treatment of BPA with MnP. After a 1-h treatment, the reaction products were eluted faster than BPA, and further coupling reaction proceeded during a 12-h treatment. The main products in the 12-h treated mixture are roughly estimated to be BPA trimers and tetramers by using dehydrogenative polymer of coniferyl alcohol (*M_w* roughly estimated to be 2500 D) and profisetinidin tetramer (approximate *M_w* 1000 D). The fact shown in Fig. 2 that 40% of the estrogenic activity of BPA remained after a 1-h treatment may be due to the degradation products from BPA keeping the structural key feature of acting as estrogens.

In this report, we showed that ligninolytic enzymes, MnP and laccase, removed the estrogenic activities of BPA and NP within 12 h and the laccase-HBT system did so

within 6 h. The mechanisms are explained by the polymerization and partial degradation of the chemicals brought about by enzymatic oxidation. To our knowledge, this is the first report dealing with the enzymatic removal of estrogenic activities of BPA and NP.

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References

- Amitai, G., Adani, R., Sod-Moriah, G., Rabinovitz, I., Vincze, A., Leader, H., Chefetz, B., Leibovitz-Persky, L., Friesem, D., Hadar, Y., 1998. Oxidative biodegradation of phosphorothiolates by fungal laccase. *FEBS Lett.* 438 (3), 195-200.
- Bourbonnais, R., Paice, M., Freiermuth, B., Bodie, E., Borneman, S., 1997. Reactivities of various mediators and laccase with kraft pulp and lignin model compounds. *Appl. Environ. Microbiol.* 63, 4627-4632.
- Bumpus, J., Tien, M., Wright, D., Aust, S., 1985. Oxidation of persistent environmental pollutants by a white rot fungus. *Science* 228, 1434-1436.
- Colborn, T., Clement, C., 1992. Chemically-induced alterations in sexual and functional development: the wildlife/human connection. In: Mehlman, M. (Ed.), *Advances in Modern Environmental Toxicology*, Vol. 21. Princeton Scientific Publishing Company, Princeton, NJ, USA.
- Deguchi, T., Kakezawa, M., Nishida, T., 1997. Nylon biodegradation by lignin-degrading fungi. *Appl. Environ. Microbiol.* 63, 329-331.
- Deguchi, T., Kitaoka, Y., Kakezawa, M., Nishida, T., 1998. Purification and characterization of a nylon-degrading enzyme. *Appl. Environ. Microbiol.* 64, 1366-1371.
- Dorn, P.B., Chou, C.S., Getempo, J.J., 1987. Degradation of bisphenol A in natural waters. *Chemosphere* 16 (7), 1501-1507.

- Ehara, K., Tsutsumi, Y., Nishida, T., 1997. Biobleaching of softwood and hardwood kraft pulp with manganese peroxidase. *Mokuzai Gakkaishi* 43, 861-868.
- Ehara, K., Tsutsumi, Y., Nishida, T., 2000. Polyethylene degradation by manganese peroxidase in the absence of hydrogen peroxide. *J. Wood Sci.* 46, 180-183.
- Iiyoshi, Y., Tsutsumi, Y., Nishida, T., 1998. Polyethylene degradation by lignin-degrading fungi and manganese peroxidase. *J. Wood Sci.* 44, 222-229.
- Johannes, C., Majcherczyk, A., Huttermann, A., 1996. Degradation of anthracene by laccase of *Trametes versicolor* in the presence of different mediator compounds. *Appl. Microbiol. Biotechnol.* 46 (3), 313-317.
- Joshi, D., Gold, M., 1993. Degradation of 2,4,5-trichlorophenol by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 59, 1779-1785.
- Kashino, Y., Nishida, T., Takahara, Y., Fujita, K., Kondo, R., Sakai, K., 1993. Biomechanical pulping using white rot fungus IZU-154. *Tappi J.* 76(12), 167-171.
- Katagiri, N., Tsutsumi, Y., Nishida, T., 1995. Correlation of brightening with cumulative enzyme activity related to lignin biodegradation during biobleaching of kraft pulp by white rot fungi in the solid-state fermentation system. *Appl. Environ. Microbiol.* 61, 617-622.
- Katagiri, N., Tsutsumi, Y., Nishida, T., 1997. Biobleaching of softwood kraft pulp by white-rot fungi and its related enzymes. *Mokuzai Gakkaishi* 48, 678-685.

- Lobos, J., Leib, T., Su, T., 1992. Biodegradation of bisphenol A and other bisphenols by a gram-negative aerobic bacterium. *Appl. Environ. Microbiol.* 58, 1823-1831.
- Nishida, T., Tsutsumi, Y., Kemi, M., Haneda, T., Okamura, H., 1999. Decolorization of anthraquinone dyes by white rot fungi and its related enzymes. *J. Jpn. Soc. Water Environ.* 22, 465-471 (in Japanese).
- Nishikawa, J., Saito, K., Goto, J., Dakeyama, F., Matsuo, M., Nishihara, T., 1999. New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. *Toxicol. Appl. Pharmacol.* 154, 76-83.
- Ollikka, P., Alhonmaki, K., Leppanen, V.-M., Glumov, T., Rajola, T., Suominen, I., 1993. Decolorization of azo, triphenyl methane, heterocyclic, and polymeric dyes by lignin peroxidase isoenzymes from *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 59, 4010-4016.
- Paice, M., Reid, I., Bourbonnais, R., Archibald, F., Jurasek, L., 1993. Manganese peroxidase, produced by *Trametes versicolor* during pulp bleaching, demethylates and delignifies kraft pulp. *Appl. Environ. Microbiol.* 59, 260-265.
- Perez, P., Pulgar, R., Olea-Serrano, F., Villalobos, M., Rivas, A., Metzler, M., Pedraza, V., Olea, N., 1998. The estrogenicity of bisphenol A-related diphenylalkanes with various substituents at the central carbon and the hydroxy groups. *Environ. Health Perspect.* 106, 167-174.
- Pickard, M.A., Roman, R., Tinoco, R., Vazquez-Duhalt, R., 1999. Polycyclic aromatic

- hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. *Appl. Environ. Microbiol.* 65 (9), 3805-3809.
- Reinhammar, B., 1984. Laccase. In: Lontie, R. (Ed.), *Copper Proteins and Copper Enzymes*, Vol. 3. CRC Press, Boca Raton, FL, pp. 1-35.
- Sato, A., Justicia, H., Wray, J.W., Sonnenschein, C., 1991. p-Nonyl-phenol: an estrogenic xenobiotic released from “modified” polystyrene. *Environ. Health. Perspect.* 92, 167-173.
- Schafer, T., Lapp, C., Hanes, C., Lewis, J., Wataha, J., Schuster, G., 1999. Estrogenicity of bisphenol A and bisphenol A dimethacrylate in vitro. *J. Biomed. Mater. Res.* 45, 192-197.
- Spivack, J., Leib, T., Lobos, J., 1994. Novel pathway for bacterial metabolism of bisphenol A. Rearrangements and stilbene cleavage in bisphenol A metabolism. *J. Biol. Chem.* 269, 7323-7329.
- Stone, R., 1994. Environmental estrogens stir debate. *Science* 265, 308-310.
- Tanghe, T., Dhooze, W., Verstraete, W., 1999. Isolation of a bacterial strain able to degrade branched nonylphenol. *Appl. Environ. Microbiol.* 65, 746-751.
- Wariishi, H., Valli, K., Gold, M.H., 1992. Manganese (II) oxidation by manganese peroxidase from basidiomycete *Phanerochaete chrysosporium*, kinetic mechanism and role of chelators. *J. Biol. Chem.* 267, 23688-23695.
- While, R., Jobling, S., Hoare, S., Sumpter, J.P., Parker, M.G., 1994. Environmentally

persistent alkylphenolic compounds are estrogenic. *Endocrinology* 135, 175-182.

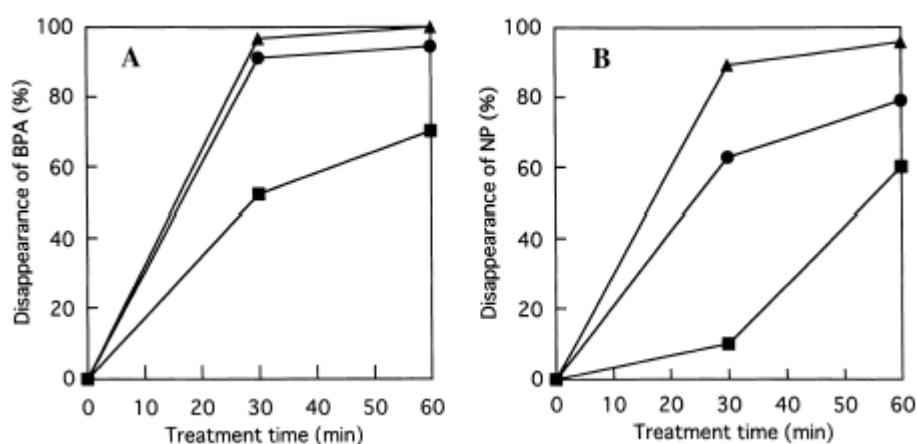


Fig. 1. Disappearance of BPA (A) and NP (B) by enzymatic treatment with MnP, laccase, or laccase-HBT system (symbols: (▲) MnP; (■), laccase; (●), laccase-HBT system).

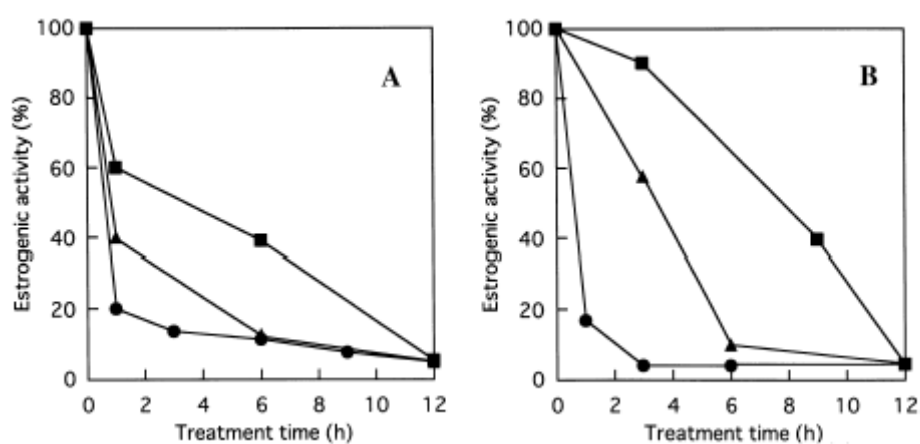


Fig. 2. Removal of estrogenic activities of BPA (A) and NP (B) by enzymatic treatment with MnP, laccase, or laccase-HBT system (symbols: (▲) MnP; (■), laccase; (●), laccase-HBT system).

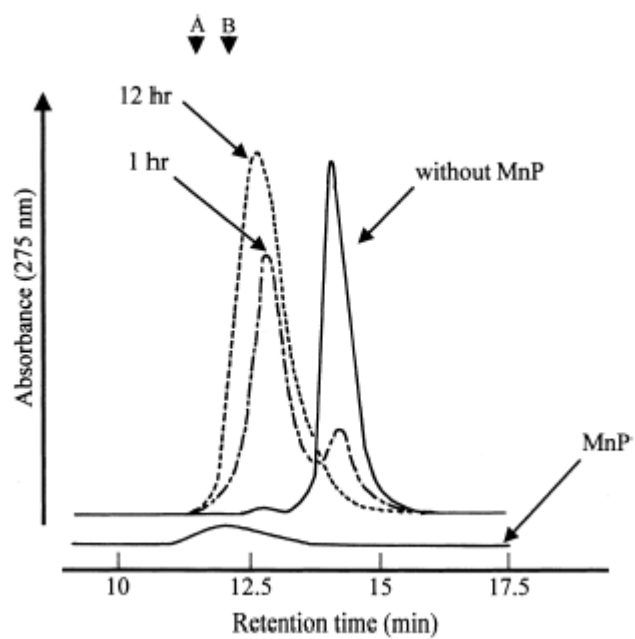


Fig. 3. GPC analysis of the reaction mixture of BPA during treatment with MnP. Marker A: dehydrogenative polymer of coniferyl alcohol (M_w roughly estimated to be 2500 D) and marker B: profisetinidin tetramer (approximate M_w 1000 D).