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Evaluation of Horseradish Peroxidase for the Treatment of Estrogenic Alkylphenols

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The xenoestrogen alkylphenols 4-nonylphenol (3.4 mg/L) and octylphenol (6.0 mg/L) were oxidized by hydrogen peroxide using horseradish peroxidase (HRP) as a biocatalyst. Substrate transformation required about one mole of peroxide per mole of phenolic compound. A high degree of conversion of alkylphenol was achieved within a 3-h reaction time. In the case of 4-nonylphenol, HRP treatment led to complete disappearance of Microtox toxicity. Results of the yeast estrogen screen (YES) assay demonstrated that the reaction products of HRP-catalyzed 4-nonylphenol conversion lacked estrogenic activity. A new approach to the YES assay has been suggested based on observations made during this study.

Key words: **peroxidase, endocrine disruptors, xenoestrogens, alkylphenols**

Introduction

Alkylphenol polyethoxylates (APnEO; AP = mainly 4-nonyl or 4-octylphenol; n = 1–18) are non-ionic surfactants used as emulsifying, dispersing, wetting and foaming agents in paints, cosmetics, agrochemicals, textile manufacturing, metal finishing, as spermicides, and as additives in lubricating oils. Biological degradation of alkylphenol polyethoxylates leads to the formation of shorter chain ethoxylates, alkylphenoxyacetic acids and finally alkylphenols (Ahel et al. 1994; Planas et al. 2002). Advanced oxidation of alkylphenol polyethoxylates, such as ozonation or photoinduced degradation by Fe(III), can also result in the formation of alkylphenols at some stages of the process (Calvosa et al. 1991; Brand et al. 1998). 4-Nonylphenol (4-NP) and 4-octylphenol (4-OP) have been shown to be highly toxic and moderately endocrine-disruptive compounds in a variety of in vivo and in vitro assays (Servos 1999). They bind to the estrogen receptor and activate the expression of estrogen-dependent genes (Tabira et al. 1999; Jørgensen et al. 2000). Due to their high hydrophobicity (e.g., log $K_{OW} \cong 4.3$; Ahel and Giger 1993b) and relative resistance to biodegradation, 4-NP and 4-OP tend to bind to particles in sediments (Isobe et al. 2001) and accumulate in living biomass (Ahel et al. 1993). Alkylphenols are more toxic than their ethoxylates and, thus, the release of these compounds via secondary effluents or sewage sludge should be avoided (Thiele et al. 1997). In a recent investigation of four Toronto sewage treatment plants, it was found that the concentration of nonylphenol ethoxylates ranged from 31 to 868 µg/L in the influents and from 3.2 to 81 µg/L in the effluents (Lee et al. 2004). Nonylphenol was present in the influent ranging from 2.8 to 16.7 µg/L and in the effluent ranging from 0.5 to 9.1 µg/L. Up to 514 µg/g of nonylphenol were detected in digested sewage sludges, on a dry weight basis (Lee et al. 2004). Collectively, these findings have raised concern over the long-term effects of these types of compounds on the reproductive health of wildlife and humans.

Although the biological degradation of alkylphenols in sewage treatment plants appears to be insufficient, it has been recently shown that 4-NP is rapidly mineralized in soil by aerobic biological degradation (Topp and Starratt 2000) and can be removed from drinking water using granulated activated carbon (U.S. EPA 2001). Other processes for the removal of endocrine disruptors from wastewaters that are currently being investigated include powdered activated carbon, ozonation and various types of membrane filtration (Filali-Meknassi et al. 2004). However, these physicochemical treatment processes generally suffer from a lack of selectivity towards target pollutants (Aitken 1993). Therefore, treatment efficiency will vary greatly depending on the characteristics of the wastewater and the presence of competing contaminants.

The aromatic structure of most estrogenic compounds (Tabira et al. 1999; Nishihara et al. 2000), including alkylphenols, indicates that they have the potential to be substrates of a group of oxidative enzymes, including various polyphenol oxidases (e.g., laccases or tyrosinase) and peroxidases (e.g., horseradish peroxidase, soybean peroxidase, etc.). Several features of enzymatic processes based on the use of these enzymes suggest that they may be particularly suited for the treatment of many endocrine-disruptive substances in effluents or in site-remediation operations. For example (Aitken 1993; Nicell 2003):

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(1) Enzymatic reactions are characterized by high substrate selectivity, high reaction rates and high stoichiometric efficiencies, which allows for efficient treatment at low pollutant concentrations. Thus, enzymes are uniquely suited for targeting trace pollutants in waters or wastewaters either as a pretreatment step or for the polishing of otherwise treated waters.
(2) Many enzymes can act on, or in the presence of, a broad range of individual chemical species or classes of compounds, including those that are bio-refractory, inhibitory or toxic.
(3) Oxidases have demonstrated their ability to operate over wide and variable ranges of temperature, pH and salinity and in the non-ideal environment found in industrial wastewaters.
(4) The high selectivity of treatment results in a low generation of by-products that must be disposed of (e.g., biomass or spent adsorbents) compared to traditional processes.
(5) Processes involving the use of isolated enzymes as catalysts are well-defined chemical systems that are more easily controlled than those based on microbial consortia in typical biological treatment systems. Therefore, enzymatic systems do not require acclimatization to a particular wastewater stream and are not susceptible to shock loading effects that may result from changes in pollutant concentration.

Recent reports have focused on evaluating the ability of certain oxidase enzymes to transform selected estrogenic compounds. For example, studies have demonstrated that the oxidative enzymes laccase and Mn-peroxidase isolated from lignin-degrading basidiomycetes were able to catalyze the conversion of estrogenic compounds including 4-NP and 17β-estradiol (Tsutsumi et al. 2001; Tanaka et al. 2000, 2001; Suzuki et al. 2003). Although reaction products were not identified, it was shown that treatment of 4-NP or 17β-estradiol led to the disappearance of the estrogenic activities within 12 or 8 h, respectively (Tsutsumi et al. 2001; Suzuki et al. 2003). These results are encouraging; however, several characteristics of these enzymes might limit their full-scale application. For example, the use of Mn-peroxidase requires the addition of Mn^{2+} ions and chelating agents, which is not practical from the point of view of wastewater treatment. Also, while the ability of these enzymes to catalyze the transformation of target compounds has been demonstrated, little information is currently available on their abilities to be catalytically active and retain activity over time in complex wastewater matrices. In addition, the nature (e.g., kinetics, mechanism of action) of these biocatalysts are not yet well understood. While the lack of information about the particular abilities and characteristics of these enzymes does not ultimately eliminate their potential for application, a significant quantity of research is required before their feasibility can be firmly established.

In contrast, horseradish peroxidase (HRP) has been extensively studied and has demonstrated its robustness as a candidate for environmental applications. HRP catalyzes the oxidation of phenols, aromatic amines and other substrates with H_2O_2 to their respective radicals, according to the following sequence of reactions (Buchanan and Nicell 1997):

$$HRP_N + H_2O_2 \rightarrow HRP_I + H_2O \quad (1)$$

$$HRP_I + ROH \rightarrow HRP_{II} + ROH \quad (2)$$

$$HRP_{II} + ROH \rightarrow HRP_N + ROH + H_2O \quad (3)$$

The native enzyme (HRP_N) is oxidized by hydrogen peroxide to an active intermediate state called compound I (HRP_I). Compound I accepts a phenolic substrate (ROH) into its active site and produces a free phenoxy radical (ROH) while being transformed into another enzyme state called Compound II (HRP_{II}). Compound II carries out a similar oxidation reaction on another substrate molecule and returns the enzyme to its native state. It should be noted that while the enzyme is a catalyst that is not consumed in the catalytic cycle, it is subject to inactivation mechanisms which limit the degree of conversion of substrate that can be achieved by a given dose of enzyme (Buchanan and Nicell 1997).

The free radicals undergo further non-enzymatic reactions leading to the formation of phenolic dimers and polymers of decreased aqueous solubility (Klibanov et al. 1980). Numerous studies have confirmed that a wide variety of compounds including chlorinated or methylated phenols, naphthols, anilines and benzidines can be treated with HRP (Klibanov et al. 1980; Klibanov and Morris 1981; Dec and Bollag 1990; Nicell et al. 1992). HRP is characterized by its high stability and its ability to catalyze the conversion of phenols over broad ranges of pH (5–9) and temperature (5–50°C) (Dec and Bollag 1990; Nicell et al. 1992, 1993). It has been shown that HRP-treatment selectively removes phenolic compounds from industrial wastewaters while improving the overall wastewater quality (Cooper and Nicell 1996; Wagner and Nicell 2001a,b). High concentrations of heavy metals, inorganic salts and a variety of organic and inorganic suspended materials were found to have little or no effect on HRP-catalyzed phenol conversion (Wagner and Nicell 2002, 2003). Certain wastewater components such as lignins were observed to make HRP-treatment more efficient in a wastewater environment than in synthetic solutions (Wagner and Nicell 2001b). However, side reactions with easily oxidizable wastewater components (e.g., sulphides) can consume some of the hydrogen peroxide that is required in the process, thereby leading to increased reagent requirements (Wagner and Nicell 2002). Protective additives such as high molecular weight PEG or chitosan can be

added to extend the enzyme's catalytic lifetime but results with real wastewaters have shown that this may not always be necessary (Wagner and Nicell 2001b). Although this technology is not yet economically attractive due to the current high cost of the enzyme, it is anticipated that recent advances in biotechnology will eventually result in a significant decrease in the cost of producing bulk quantities of enzymes.

The ultimate goal of this work is to evaluate whether the HRP-based process could also be used for the treatment of estrogenic compounds. Alkylphenols were selected for this particular study because they have been widely recognized to be compounds of concern. For example, the European Economic Community has proposed that the maximum limits of 4-nonylphenol and its ethoxylates be 50 mg/kg in sludge that is to be used on agricultural land. In addition, in Canada it has been recommended that these compounds be added to the List of Toxic Substances under the *Canadian Environmental Protection Act* (Canada Gazette 2001). The specific objectives were: (1) to investigate whether the alkylphenols 4-NP and 4-OP can be treated with HRP and H_2O_2 and (2) to determine the impact of the enzymatic treatment on the Microtox acute toxicity and the estrogenicity of the reaction mixtures. The yeast estrogen screen (YES) reporter gene assay, as developed by Gaido et al. (1997), was used to measure estrogenic effects.

Materials and Methods

Chemicals

Horseradish peroxidase HRP (EC 1.11.1.7, RZ 1.3) was purchased from Sigma-Aldrich Chemical Co., St Louis, Mo. The nominal activity of HRP was quoted by the supplier as being 116 units/mg dry solid, where one unit of activity corresponds to the formation of 1 mg purpurogallin from pyrogallol in 20 seconds at 20°C and pH 6. HRP stock solutions (5 mg/mL) were prepared by dissolving the solid enzyme in distilled deionized water and were stored at 4°C. Hydrogen peroxide (30 % w/w) and 4-aminoantipyrine (4-AAP) (98% purity) were purchased from Aldrich Chemical Co., Milwaukee, Wisc. Phenol (99.5%+ purity) was purchased from Fluka Chemical Corporation, Ronkonkona, N.Y.

Nonylphenol (4-NP; Riedel-de Haen, technical mixture, 94% purity) and 4- octylphenol (4-OP; Aldrich, 99% purity), L-histidine-HCl-H_2O, L-lysine-HCl, cupric sulfate pentahydrate (>98%; plant cell culture tested), *o*-nitrophenyl β-D-galactopyranoside (ONPG; >98%), lauryl sulfate sodium salt (SDS; >99%), 17β-estradiol (>98%) and β-mercaptoethanol were purchased from Sigma-Aldrich Chemical Co., St. Louis, Mo. Yeast nitrogen base without amino acids was manufactured by Becton, Dickinson and Company, Md. Dextrose (A.C.S. reagent) was purchased from J.T. Baker Inc., Phillisburg, N.J. All buffer salts were of analytical quality and they were obtained from Anachemia Canada Inc. or Fisher Scientific Co., Montreal. Oxalyticase (50,000 units/mg) was acquired from Enzogenetics, Corvallis, Oreg.

The *Saccharomyces cerevisiae* strain BJ3505 hER 2ERE from Dr. K.W. Gaido [MATα, pep4::HIS3, prb1-Δ1.6*R*, his3-Δ200, lys2-801, trp-Δ101, ura3-52 (can)] was kindly donated by Dr. B. Kent Burnison, Environment Canada, National Water Research Institute, Burlington, Ontario.

HRP Activity Assay

The activity of the HRP stock solution (5 mg of dry solid/mL) was measured using a colorimetric assay containing 2.4 mM 4-AAP, 10 mM phenol and 0.2 mM H_2O_2 in 0.1 M phosphate buffer at pH 7.4. Prior to significant substrate depletion, activity is proportional to the rate of formation of a precipitating red antipyrilquinoneimine dye that absorbs light at a peak wavelength of 510 nm with an extinction coefficient of 7100 M^{-1} cm^{-1} based on the conversion of H_2O_2 (Nicell and Wright 1997). One unit of activity is defined as the number of micromoles of H_2O_2 consumed per minute at pH 7.4 and 25°C.

Enzymatic Treatment

4-OP and 4-NP stock solutions were prepared in methanol and stored at 4ºC. Alkylphenol reaction mixtures were made by adding the appropriate volumes of stock solutions into distilled-deionized water, which resulted in less than 0.7% of methanol in the aqueous phase. Reactions were carried out in 20-mL borosilicate glass vials or 100-mL glass beakers and the reaction volumes were 10 and 100 mL, respectively. Reactions were initiated by the addition of aliquots of enzyme (5 mg/mL) and H_2O_2 (5–10 mM) stock solutions. The reaction mixtures were briefly mixed and allowed to incubate for 3 h at room temperature (20°C) before HPLC analysis or solid-phase extraction was done. During the reaction the glass vials and beakers remained sealed with screw caps or parafilm, respectively.

RP-HPLC

The concentrations of 4-NP and 4-OP in water or in methanol were determined through RP-HPLC using Agilent 1100 Series HPLC. The column was a Vydac C18 bonded to TP (300 Å) silica (150 mm long × 2.1 mm i.d., p.d. 5 μm). The mobile phase consisted of 45% water and 55% acetonitrile and the flow rate was 0.8 mL/min. Detection was carried out using fluorescence detection at an excitation wavelength of 223 nm and emission wavelength of 317 nm. The column temperature was kept constant at 22ºC.

Acute Toxicity

Acute toxicity was determined using the 5-min Microtox assay with the Model 500 Toxicity Analyzer according to the Basic Test procedure recommended by the instrument manufacturer (Azur Environmental, Carlsbad, Calif.). Ghioureliotis and Nicell (1999) described this procedure and its application to enzymatic treatment studies in detail. The Microtox assay involves the short-term incubation of the bioluminescent marine microorganism *Photobacterium phosphoreum* in a series of dilutions of the sample. Light output is reduced in proportion to the degree of toxicity of the sample. The concentration of sample (expressed as a volume fraction in the assay) that causes a 50% reduction in light output is defined as the effective concentration, EC_{50}, of the sample. The quality of the measurements was verified by assaying a standard phenol solution.

Solid-Phase Extraction

Solid-phase extraction of aqueous 4-NP samples was carried out using Supelclean LC-18 SPE tubes of 0.5-g packing and 6-mL capacity (Supelco, Sigma-Aldrich, Chemical Co., St. Louis, Mo.). After conditioning with methanol and water, the sample (100 mL) was passed through the tube at a rate of less than 5 mL/min. The tube was washed with water and dried in air. Retained compounds were eluted with 2 mL of methanol and the eluate was filtered through a syringe filter (regenerated cellulose, pore size 0.45 µm) before HPLC analysis. Thus, solid-phase extraction into methanol resulted in about 50-fold sample concentration. After solid-phase extraction, approximately 75% of the 4-NP was recovered in samples that had been treated with the enzyme only (i.e., where there was a high residual 4-NP). 4-NP recovery rates were 100% in samples with low residual 4-NP that had been treated with HRP and H_2O_2.

Yeast Estrogen Screen (YES) Assay

The yeast (*Saccharomyces cerevisiae*) strain developed by Gaido et al. (1997) carries an expression vector encoding the human estrogen receptor where its expression is under the control of the CUP1 metallothionein promotor and a reporter gene vector composed of an estrogensensitive promotor linked to the reporter gene β-galactosidase. Binding and activation of the human estrogen receptor by an estrogenic compound induces the expression of β-galactosidase. Analysis of β-galactosidase activity involves the release of the enzyme by cell lysis and the addition of the chromogenic substrate *o*-nitrophenyl β-D-galactopyranoside. The formation of the reaction product *o*-nitrophenol is followed by the increase in the absorbance at 420 nm.

The assay was carried out essentially following the procedures of Gaido et al. (1997) and Hoekstra et al. (2001). Yeast cells were grown overnight with vigorous orbital shaking at 250 rpm and 30°C in selective synthetic dextrose minimal medium (SD-MM+His+Lys – buffered) containing 6.7 g/L Yeast Nitrogen Base without amino acids, 20 g/L glucose, 24 mg/L histidine-HCL, 36 mg/L lysine-HCL dissolved in 0.05 M sodium phosphate buffer and pH 6.8. Cells were subcultured the next day and allowed to grow to mid-log phase (OD_{600} = 0.3 to 0.4). After 10-fold dilution with medium and the addition of filter-sterilized $CuSO_4$ at a concentration of 0.1 mM the solution was dispensed at 5 mL into acid-washed 50-mL glass centrifuge tubes. Five microlitres of sample dissolved in methanol or methanol blanks were added to each tube. The assay was carried out in triplicate. The yeast cultures were incubated overnight at 30°C and 250 rpm.

The following day, yeast cultures were assayed for β-galactosidase activity. Eight hundred microlitres of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl and 1 mM $MgSO_4$, pH 7.0) containing 43.75 mM β-mercaptoethanol and 0.125% SDS, 100 µL of oxalyticase solution (1000 units/mL Z-buffer), 100 µL of yeast sample and 100 µL of ONPG substrate solution (8 mg/mL Z-buffer) were placed into a 1-mL cuvette in that order. The cuvette was inverted twice, placed into the spectrophotometer and the absorbance at 420 nm (*o*-nitrophenol product) and at 600 nm (light scatter) was recorded for 150 s. The β-galactosidase activity for each sample was determined using the following equation:

$$\beta-\text{galactosidase activity (min}^{-1}) = 1000 \times \frac{d(A_{420} - A_{600})}{dt} \times \frac{1}{A_{600\,\text{initial}}} \times \frac{60\text{s}}{\text{min}} \quad (4)$$

A Hewlett Packard 8453 diode array spectrophotometer (wavelength range 190–1100 nm with 2-nm resolution) was used for all absorbance measurements.

Results

Treatment of 4-Nonylphenol and 4-Octylphenol with Horseradish Peroxidase

To begin, tests were carried out to establish whether the alkylphenols 4-NP and 4-OP could be oxidized by H_2O_2 using HRP as a biocatalyst. Figure 1 shows the results of the treatment of aqueous 4-NP (3.5 mg/L) and 4-OP (6.0 mg/L) with HRP and/or H_2O_2. No 4-NP or 4-OP removal was observed when either the peroxidase or the H_2O_2 were omitted from the reaction mixture. Thus, the disappearance of the alkylphenols was due to a combined action of HRP and H_2O_2, thereby indicating that these compounds were oxidized under the catalytic action of HRP.

Further experiments were performed to determine how much H_2O_2 would be required to accomplish the

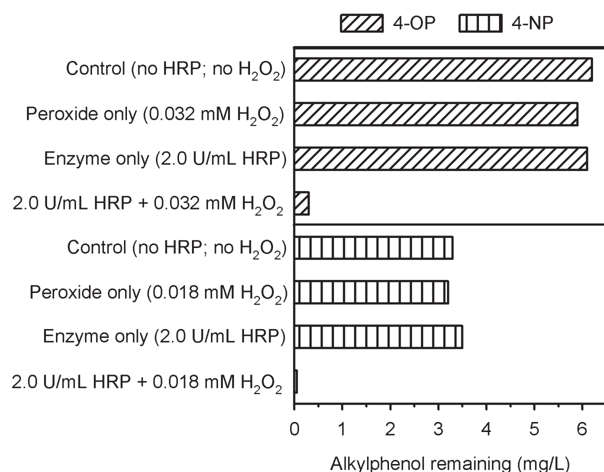

Fig. 1. Residual 4-octylphenol (4-OP) and 4-nonylphenol (4-NP) remaining after treatment with HRP/H_2O_2 with a retention time of 3 h and a reaction temperature of 20°C. Residual concentrations in control samples (no HRP; no H_2O_2) were the same as initial concentrations.

conversion of 4-NP or 4-OP at a constant enzyme dose of 2.0 U/mL. The results are shown in Fig. 2. The concentration of the alkylphenols decreased linearly with increasing H_2O_2 dose. On average, 1.01 ± 0.12 and 1.16 ± 0.09 moles H_2O_2 were required for the conversion of 1 mole of 4-NP and 4-OP, respectively.

Characterization of Reaction Products

Aqueous 4-NP solutions (i.e., 1.5×10^{-5} M or 3.4 mg/L dissolved in distilled-deionized water) were treated with 2.0 U/mL of HRP and 0.0166 mM H_2O_2 or with 2.0 U/mL of HRP alone in the absence of H_2O_2 for three hours at room temperature. Treatment with peroxidase and H_2O_2 resulted in 93% 4-NP removal. No 4-NP conversion occurred in the absence of H_2O_2.

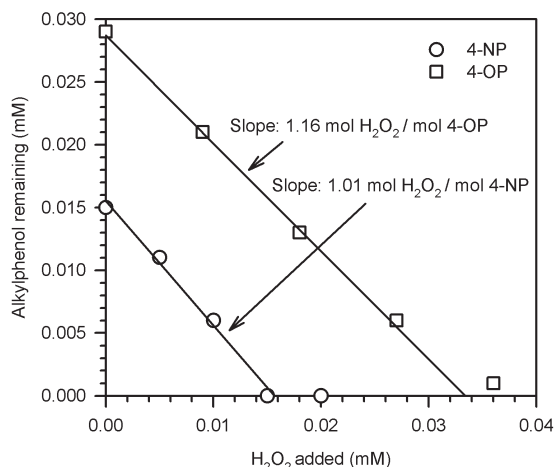

Fig. 2. 4-Octylphenol (4-OP) and 4-nonylphenol (4-NP) remaining as a function of the H_2O_2 concentration supplied to the reaction mixture with a retention time of 3 h and a reaction temperature of 20°C.

Previous studies have shown that, depending on the type of phenol, HRP-catalyzed phenol conversion in aqueous solutions can fail to reduce toxicity and can even increase it (Aitken et al. 1994; Ghioureliotis and Nicell 2000). This phenomenon was generally attributed to the formation of trace amounts of soluble reaction products that were more toxic than the parent substrate (Ghioureliotis and Nicell 1999, 2000). Therefore, the effect that HRP-catalyzed conversion of 4-NP had on toxicity levels was examined.

The toxicities of the aqueous samples were measured using the Microtox acute toxicity assay. A 1.5×10^{-5} M solution of 4-NP, dosed with 2.0 U/mL of HRP, had a TU_{50} of 6.3 ± 0.8. This means that it needed to be diluted 6.3 times in order to decrease the luminescence of the Microtox organism by 50%. This is equivalent to an EC_{50} of $(2.5 \pm 0.3) \times 10^{-6}$ M or 0.54 ± 0.06 mg/L 4-NP, which, on a mass basis, is approximately 37 times more toxic than phenol. Previous experiments had confirmed that the enzyme does not contribute to toxicity in the Microtox assay and, thus, these results reflect toxicity due to 4-NP only. Samples that had been treated with both enzyme and H_2O_2 failed to produce any toxic effect (i.e., no decrease in light output of the chemiluminescent bacteria) in the Microtox assay even at a dilution of 0.45, which is the lowest dilution that can be tested according to the standard test protocol. Thus, it can be concluded that there was no significant amount of toxic compounds present in the samples after enzymatic conversion of 4-NP and that enzymatic treatment accomplishes a high degree of detoxification.

The YES assay was used in order to determine whether the products of the enzymatic conversion of aqueous 4-NP would have estrogenic potential. Figures 3A and 3B show the percentages β-galactosidase induction produced by 17β-estradiol and 4-NP, respectively, as a function of concentration in the assay. The level of β-galactosidase induction generated by 17β-estradiol increased linearly up to a concentration of 4×10^{-10} M and then flattened off as the concentration approached 10^{-9} M. The level of induction occurring at 10^{-9} M 17β-estradiol was designated as 100%. In the case of 4-NP, β-galactosidase induction increased linearly up to a concentration of 3.5×10^{-7} M and reached 100% (corresponding to 10^{-9} M 17β-estradiol) at 10^{-6} M (Fig. 3B). The concentrations of 17β-estradiol and 4-NP at which half-maximum β-galactosidase induction occurred (EC_{50}) were 3.9×10^{-10} M and 2.8×10^{-7} M, respectively. The half-maximum value is often used as a means of comparing the relative estrogenicities of compounds (Hoekstra et al. 2001; Beresford et al. 2000). On this basis, 17β-estradiol is approximately 718 times more estrogenic than 4-NP; thus a 1.5×10^{-5} M 4-NP solution corresponds to 2.1×10^{-8} M 17β-estradiol.

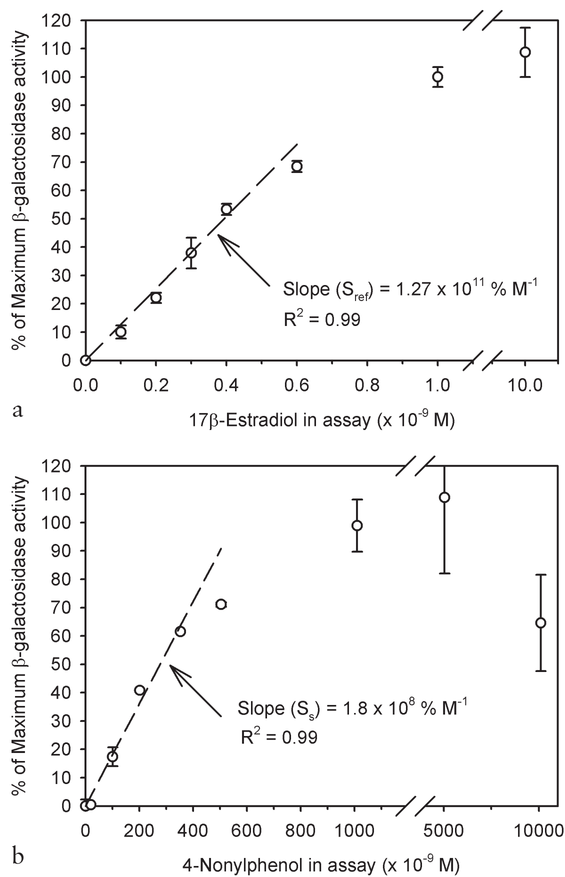

Fig. 3. YES assay response (% of the maximum β-galactosidase activity) as a function of the (A) 17β-estradiol (averages and standard errors of five independent experiments are shown) and (B) 4-NP concentrations in the assay (averages and standard errors of three independent experiments are shown).

Aqueous samples of 1.5×10^{-5} M 4-NP were treated with HRP only (no H_2O_2) or with HRP and H_2O_2. No change in 4-NP concentration was recorded in samples treated with HRP only. In samples treated with both reagents, the 4-NP concentration dropped to 0.12×10^{-5} M (92.3% removal), which theoretically corresponds to 1.7×10^{-9} M 17β-estradiol. The estrogenic effects of these samples were quantified using the YES assay. In Fig. 4, the estrogenic responses of both samples are plotted as a function of the 4-NP concentration in the assay solution. The 17β-estradiol and 4-NP standard plots are included for reference. From this plot, it can be seen that the estrogenic effects of both samples closely corresponded to their 4-NP concentrations. In fact, the HRP/H_2O_2-treated sample produced the same estrogenic effect as $(1.6 \pm 0.2) \times 10^{-9}$ M 17β-estradiol (after taking the dilution into account), which is very close to the theoretical value of 1.7×10^{-9} M. These results indicate that the products from the peroxidase-catalyzed conversion of 4-NP lack estrogenic activity.

RP-HPLC analysis of both the aqueous sample and the methanol extract and GC/MS analysis of the methanol extract failed to detect the presence of 4-NP reaction products, further indicating that these products were highly insoluble, which is in agreement with the results of both the residual toxicity and estrogenicity measurements.

Discussion

This study has demonstrated that aqueous 4-NP and 4-OP can be treated with HRP and H_2O_2, resulting in a high degree of phenol conversion; i.e., a dose of 2.0 U/mL of HRP achieved 93% conversion in a 3-h reaction time.

Treatment required about one mole of H_2O_2 per mole of phenolic compound. This value is in good agreement with the 1-to-1 stoichiometry observed for HRP-catalyzed conversion of eight phenolic compounds (Nicell et al. 1992). The apparent 1-to-1 stoichiometry, which is in contrast to the theoretical 2-to-1 phenolic substrate-to-peroxide stoichiometry predicted by the reactions in equations 1, 2 and 3, has been attributed to the participation of phenolic dimers or other oligomers in the catalytic cycle, which react to form even larger oligomers (Hewson and Dunford 1976; Nicell et al. 1992). In contrast, when pentachlorophenol (PCP) was treated with HRP and H_2O_2 0.5 moles of H_2O_2 were required for the oxidation of 1 mole of pentachlorophenol, indicating that pentachlorophenoxy radicals react to a compound that cannot further be oxidized with HRP (Zhang and Nicell 2000). This compound has been identified as an insoluble dimer lacking reactive hydroxyl groups (Kazunga et al. 1999). Thus, the observed 1-to-1 stoichiometry between the alkylphenols and H_2O_2 suggests that oligomeric products of HRP-catalyzed alkylphenol oxidation were further metabolized to higher molecular weight compounds. Similarly, Tsutsumi et al. (2001) reported that Mn-peroxidase

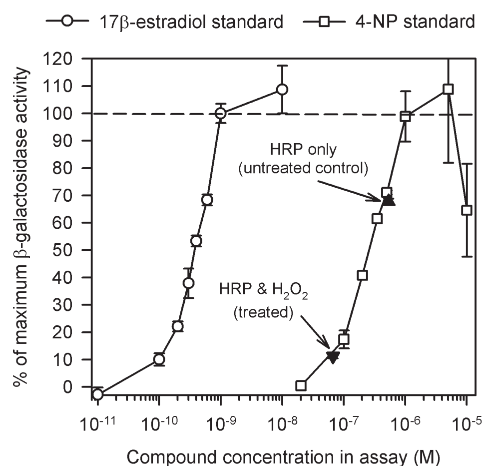

Fig. 4. β-Galactosidase activity induced by 4-nonylphenol samples treated with 2.0 U/mL of HRP and 0.0166 mM H_2O_2 or with 2.0 U/mL of HRP alone as a function of their residual 4-NP concentration compared with the assay's response to 17β-estradiol and 4-NP standards. Averages and standard errors of two independent experiments are shown.

or laccase treatment of 4-NP resulted in high molecular weight reaction products.

In previous work, it had been demonstrated that with certain substrates, including phenol, there was an increase in toxicity that resulted from the accumulation of low concentrations of sparingly soluble reaction products (e.g., dimers and trimers) in the aqueous phase after treatment (Ghioureliotis and Nicell 1999, 2000). In the present case, the aqueous solubility of the substrate 4-NP is very low (5.43 mg/L or 0.0247 mM) at 20.5°C (Ahel and Giger 1993a). Thus, it can be expected that any dimers or oligomers formed from 4-NP conversion would have extremely low solubility and, as a result, would not tend to exert significant toxicity in the Microtox assay.

Results from the YES assay suggest that the products from the peroxidase-catalyzed conversion of 4-NP lack estrogenic activity. In contrast, a previous study has shown that when treatment of 4-NP solutions was conducted with Mn-peroxidase or laccase, 60% or 90% of the estrogenic activity remained after 3 h, respectively, even though complete 4-NP conversion had been achieved at that point in time (Tsutsumi et al. 2001). The estrogenic activity of 4-NP solutions was almost entirely removed when Mn-peroxidase or laccase treatments were extended to 12 h (Tsutsumi et al. 2001). The differences between these results and those presented here could be due to differences in catalytic characteristics of the various enzymes. Also, the concentration of 4-NP used by Tsutsumi and coworkers (2001) was 10 times higher than in the present study, which necessitated the addition of Tween 80 to enhance its aqueous solubility.

Assays similar to the YES assay used here were previously applied to determine the estrogenic activity of pure compounds and environmental samples (Legler et al. 2002; Beresford et al. 2000; Hoekstra et al. 2001). However, the characteristics of the dose-response curves shown in Fig. 3 indicate that EC_{50}s are not necessarily the ideal parameters for comparing estrogenic effects of known compounds or assaying estrogenic activities of unknown samples. Firstly, the assay response becomes highly variable close to the level of the maximum β-galactosidase induction (i.e., above 17β-estradiol concentrations of 10^{-9} M or 4-NP concentrations of 10^{-6} M). This variability has also been observed in other studies (e.g., Gaido et al. 1997). The pronounced variability of the assay response at the higher estrogen concentrations coincided with reduced cell densities, as reflected by a significant reduction in the OD_{600} values of the yeast cultures, and suggested the presence of inhibitory or toxic effects of the test compound on the assay organism. This variability introduces a high degree of uncertainty in the determination of the level of the maximum response, even for the 17β-estradiol which is used as a standard, thereby posing a problem for the accurate and repeatable determination of EC_{50}s. Secondly, many compounds or environmental samples fail to produce the maximum or sometimes even a 50% response in the assay (Hoekstra et al. 2001; Beresford et al. 2000; Routledge and Sumpter 1996), possibly due to toxic or inhibitory effects. Even if a compound induces a 100% response, its toxicity to the assay organism is likely to influence the shape of the dose-response curve in the higher concentration range, as can be suspected in the case of 4-NP, which exhibits acute Microtox toxicity ($EC_{50} = 2.5 \times 10^{-6}$ M) at concentrations that have strong estrogenic effects. Thirdly, at the 50% response the assay is approaching saturation. As a result, each additional molecule of estrogenic compound has a lesser impact on estrogenic response than previous molecules. Thus, the assay response becomes confounded by the lack of availability of receptors to which the estrogenic compound could bind.

To reduce these problems, a revised approach is suggested here to determine relative estrogenic potencies using a YES assay. When the estrogenic response is measured under dilute conditions, toxicity is minimized and the assay is far from saturation conditions. Under these conditions, each additional molecule of estrogenic compound should have a proportional impact on the assay response, resulting in a linear dose-response relationship, as shown in Fig. 3 for low concentrations of 17β-estradiol and 4-NP. When a linear dose-response relationship is obtained, it is proposed that the estrogenicity of a compound or an environmental sample expressed relative to a standard, i.e., relative estrogenicity (RE), can be expressed by:

$$RE = \frac{S_s}{S_{ref}} \tag{5}$$

where S_{ref} is the linear slope for the reference standard (e.g., 17β-estradiol, see Fig. 3A) standard in % M^{-1} and S_s is the linear slope for a sample in % M^{-1} (for samples of pure compounds with known concentration; e.g., see Fig. 3B) or in % (for environmental samples of unknown composition). Note that RE is expressed in units of moles of the reference standard per mole sample compound (i.e., dimensionless), in the case of pure compounds, or in molar equivalents of the reference standard in the case of environmental samples whose estrogenic compound(s) are unidentified or not quantified.

The proposed method will be illustrated by applying it to a sample. First, a range of dilutions of the sample should to be prepared of sufficiently low estrogenicities to ensure that the assay responses used for slope evaluation over a range of dilutions will fall into the linear range. The assay response is recorded and plotted as a function of 1/D, where D is the dilution factor (D, dimensionless), defined as:

$$D = \frac{V_s + V_D}{V_s} \tag{6}$$

where V_s is the sample volume and V_D is the volume of diluent. Note that the inverse term of 1/D is proportional to the concentration of the dissolved species.

An example of this type of graph is shown in Fig. 5. It can be seen that the first four dilutions of the sample give a relatively linear response and that the y-intercept is near zero. The slope of the straight line passing through these points (i.e., S_s) is 2.70×10^3 %. From our 17β-estradiol standard plot shown in Fig. 3A, the value of $S_{ref} = 1.27 \times 10^{11}$ % M^{-1} is obtained for 17β-estradiol as the reference material. Thus, by substituting these values into equation 5, the relative estrogenicity (RE) for this sample is calculated to be 2.13×10^{-8} M equivalents as 17β-estradiol.

Upon analysis, it was determined that the undiluted sample contained 1.5×10^{-5} M of 4-NP as the sole estrogenic compound. In this case, with the concentration of the sole estrogenic compound known, the 1/D values plotted in Fig. 5 could be replaced with accurate 4-NP concentrations and a plot similar to that shown in Fig. 3B could be obtained. For this sample, a plot of maximal activity versus 4-NP concentration (not shown) gives a slope (i.e., S_s) of 1.80×10^8 % M^{-1} 4-NP. According to equation 5 the RE of 4-NP in this sample is then 1.42×10^{-3} mol 17β-estradiol/mol 4-NP. The inverse of this value is 706 mol 4-NP/mol 17β-estradiol. Thus, based on this approach, 17β-estradiol is about 706 times more potent than 4-NP under the assay conditions. It is not surprising that this value is similar to the value of 718 quoted above (see the Characterization of Reaction Products section) which was based on the ratios of the EC_{50}s of the compounds, since the half-maximum responses for both 17β-estradiol and 4-NP fell into the linear range as shown in Fig. 3A and 3B. However, this would not necessarily be the case for other compounds or environmental samples. In such cases, because the proposed method uses only data collected in the linear region, it is less subject to problems that emerge at higher assay concentrations.

In this study, the YES and Microtox assays were used as screening tools to assess treatment efficiency with respect to water quality parameters other than simply the degree of transformation of the target compound. It should be noted that a wider array of estrogenicity and toxicity tests, including those involving potential target organisms, is required in order to fully evaluate the potential environmental impacts of the treated wastewaters.

Conclusions

This study has demonstrated that aqueous 4-NP and 4-OP can be treated with HRP and H_2O_2. Treatment required about 1 mole H_2O_2 per mole phenolic compound and more than 93% transformation was achieved within a 3-h reaction time. In the case of 4-NP, HRP treatment led to complete disappearance of Microtox toxicity. The results of the YES assay demonstrated that the reaction products of HRP-catalyzed 4-NP conversion, as far as they were transferred through solid-phase extraction into methanol, lack estrogenic activity. In addition, as an outcome of this study, a revised method to determine relative estrogenic potencies of compounds and environmental samples from results of the YES assay was proposed. Based on these findings and the proven ability of HRP to operate under a variety of conditions and in real wastewater environments, the HRP-based process may become a viable option for the treatment of waters containing estrogenic alkylphenols and other estrogenic compounds. To explore this option further, research should aim at treating other types of estrogenic compounds, identifying and characterizing reaction products, and establishing the amounts of enzyme required for treatment.

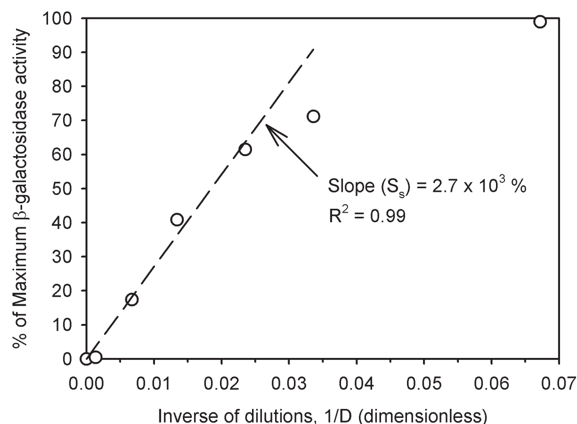

Fig. 5. β-Galactosidase activity induced by an estrogenic sample as a function of the inverse of the dilution factor (1/D). Sample contained 1.5×10^{-5} M of 4-nonylphenol.

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