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ORIGINAL PAPER

Oxidation of pharmaceutically active compounds by a ligninolytic fungal peroxidase

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Abstract Pharmaceuticals are an important group of emerging pollutants with increasing interest due to their rising consumption and the evidence for ecotoxicological effects associated to trace amounts in aquatic environments. In this paper, we assessed the potential degradation of a series of pharmaceuticals: antibiotics (sulfamethoxazole), antidepressives (citalopram hydrobromide and fluoxetine hydrochloride), antiepileptics (carbamazepine), anti-inflammatory drugs (diclofenac and naproxen) and estrogen hormones (estrone, 17β-estradiol, 17α-ethinylestradiol) by means of a versatile peroxidase (VP) from the ligninolytic fungus *Bjerkandera adusta*. The effects of the reaction conditions: VP activity, organic acid concentration and H_2O_2 addition rate, on the kinetics of the VP based oxidation system were evaluated. Diclofenac and estrogens were completely degraded after only 5–25 min even with a very low VP activity (10 U l^{-1}). High degradation percentages (80%) were achieved for sulfamethoxazole and naproxen. Low or undetectable removal yields were observed for citalopram (up to 18%), fluoxetine (lower than 10%) and carbamazepine (not degraded).

Keywords Pharmaceutical xenobiotics · Estrogens · Enzymatic oxidation · Versatile peroxidase (VP)

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Introduction

There is increasing concern about potential ecological and adverse health effects resulting from the use of numerous chemicals with beneficial purposes for human life. Nearly 3,000 household chemicals, pharmaceuticals and other consumables as well as biogenic hormones are registered in the European Union (Joss et al. 2006). These compounds are partially absorbed by human metabolism and later excreted with identical or altered physico-chemical properties. The discharge of these recalcitrant compounds in the environment represents an important ecological impact since their complex structure confers resistance and impedes their biodegradation in conventional wastewater treatment plants (Carballa et al. 2004). Furthermore, they are potentially toxic on aquatic and terrestrial organisms, since they are deliberately designed to obtain a biological effect even at very low concentrations. Some of them may even accumulate in biota. Therefore, environmental engineering faces an emerging issue: the effective removal of pharmaceutical compounds from sources

such as hospital and urban wastewaters before their discharge. Since conventional wastewater treatment processes were demonstrated to be inefficient for the degradation of some recalcitrant pharmaceuticals, the implementation of advanced treatment technologies in areas where a persistent pollution problem has been recognized is required. Several advanced treatment technologies (including ozone treatment) have been evaluated for this purpose in recent years, obtaining different outcomes (Ikehata et al. 2006).

In an attempt to overcome some of the deficiencies related to traditional chemical and biological treatment processes (Eibes et al. 2007), the use of enzymes isolated from their parent organism is proposed to catalyze the transformation of target pollutants. Among them, oxidative enzymes, including polyphenol oxidases (e.g., tyrosinase, laccases) or hydrogen peroxide oxidoreductases (e.g., peroxidases from horseradish, soybeans, etc.) have been evaluated for the treatment of wastewaters (Duran and Esposito 2000). It has recently been demonstrated that manganese peroxidase (MnP), horseradish peroxidase and laccase are effective in removing the estrogenic activities of endocrine disrupting chemicals such as bisphenol A, nonylphenol, estrone, 17β-estradiol and ethinylestradiol (Tsutsumi et al. 2001; Suzuki et al. 2003; Tamagawa et al. 2006; Cabana et al. 2007; Auriol et al. 2008), that lignin peroxidase (LiP) can attack the anti-inflammatory diclofenac (Zhang and Geißen 2010), and that the laccase-mediator system can remove carbamazepine (Hata et al. 2010b).

Versatile peroxidase (VP) is a heme peroxidase produced by a few white rot fungi. It is capable of oxidize Mn^{2+} as well as non-phenolic aromatic compounds in the presence of hydrogen peroxide (H_2O_2) (Mester and Field 1998; Palma et al. 2000). The generated Mn^{3+}, which diffuses away from the enzyme active site and is chelated by a dicarboxylic organic acid, sequentially oxidizes a compound that may or may not be substrate of the enzyme. The enzyme is returned to its original form and can subsequently be used to accomplish the oxidation of more compounds. Examples of compounds oxidized by VP are aromatic polycyclic hydrocarbons (Eibes et al. 2006) or industrial dyes (Mielgo et al. 2003) in the presence of low concentrations of Mn^{2+}.

The objective of this research was to determine the effect of reaction conditions on the effectiveness of VP-catalyzed oxidation on pharmaceutical compounds in an enzymatic reactor. Several structurally diverse pharmaceuticals (Table 1) belonging to the most important medicine classes were chosen for this study: one antibiotic (sulfamethoxazole), two anti-depressants (citalopram, fluoxetine), one anti-epileptic (carbamazepine), two anti-inflammatory drugs (diclofenac, naproxen), one tranquillizer (diazepam) and estrogen hormones (estrone, 17β-estradiol, 17α-ethinylestradiol). These compounds represent a wide range of commonly prescribed therapeutic classes, with different physico-chemical properties and potential severe effects on the environment (like antibiotics and anti-depressants). They all have been detected in wastewater treatment plants, rivers or groundwater at different concentrations ranging from nanograms to micrograms per litre (Ternes 1998; Carballa et al. 2004; Ternes and Joss 2006). In the present article, the enzymatic removal of pharmaceuticals is evaluated at much higher concentrations than those reported (milligrams per litre) in order to simulate a contaminated site such as hospital wastewaters. The degradation kinetics of the pharmaceuticals were evaluated under different reaction conditions, including enzymatic activity levels, organic acid concentration and H_2O_2 addition rate.

Materials and methods

Chemicals and enzyme

Sulfamethoxazole (SMX), citalopram hydrobromide (CTL), fluoxetine hydrochloride (FLX), carbamazepine (CBZ), diclofenac (DCF), naproxen (NPX), estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2) and 3-amino-5-methylisoxazole (AMI) were purchased from Sigma–Aldrich with purities higher than 98%. H_2O_2 (30% v:v), sodium malonate (>97%) and manganese sulphate (>98%) were also from Sigma–Aldrich. Acetone and acetonitrile were obtained from Panreac (chemical purity). Stock solutions of the diverse compounds (250 mg l^{-1}) were prepared in acetone.

VP was obtained from *Bjerkandera adusta* (ATCC 90940). The fungus was grown in a 10 l fermenter BIOSTAT-E (B. Braun-Biotech International), on skimmed cheese whey medium (Feijoo et al. 1999). Once the peak production of VP was detected, fermentation was stopped by decreasing temperature

Table 1 Physico-chemical properties of the selected pharmaceuticals and hormones

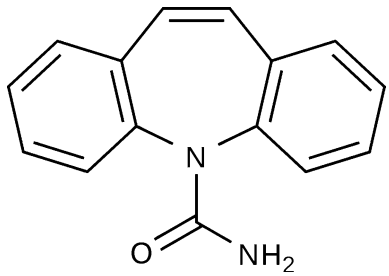
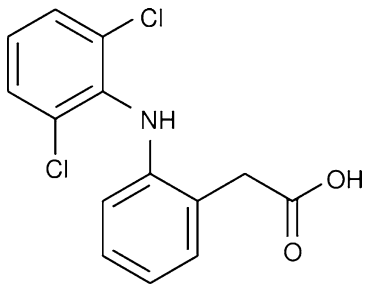
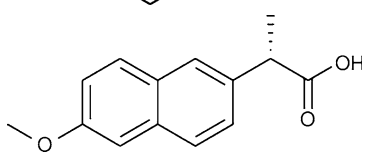
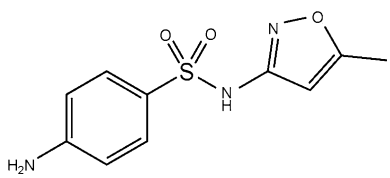
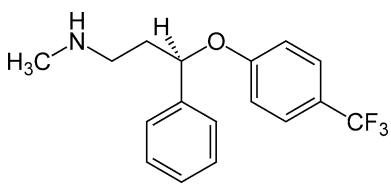
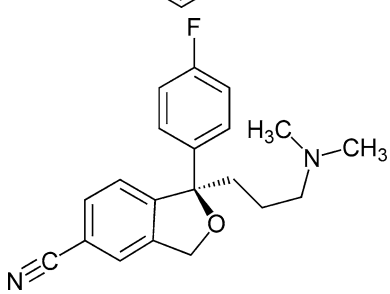
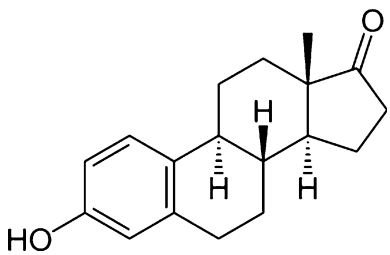
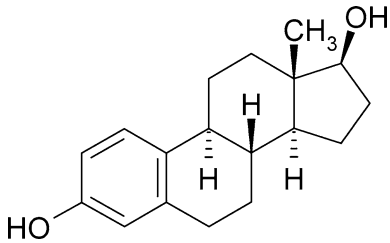
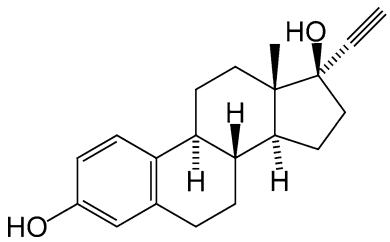
Pharmaceutical	Structure	Molecular weight (Da)	Water solubility (mg l^{-1})	pKa	Log K_{OW}
Antiepileptic					
Carbamazepine		236.3	17.7	13.9	2.5–3.0
Analgesics					
Diclofenac		318.1	2.4	4.0–4.5	4.5–4.8
Naproxen		230.3	16	4.2	4.2
Antibiotics					
Sulfamethoxaxole		253.3	610	5.6–6.0	0.5–0.9
Antidepressives					
Fluoxetine hydrochloride		309	60	10.1	4.05
Citalopram hydrobromide		324	31	9.6	2.9–3.7

Table 1 continued

Pharmaceutical	Structure	Molecular weight (Da)	Water solubility (mg l^{-1})	pKa	Log K_{OW}
Estrogens					
Estrone		270.4	30	10.4	3.1–3.4
17β-estradiol		272.4	3.6	10.4	3.9–4.0
17α-ethinylestradiol		296.4	11.3	10.5–10.7	2.8–4.2

and the extracellular liquid was vacuum filtered. High molecular weight polysaccharides were precipitated by freezing-thawing and removed by filtration. Crude enzyme was concentrated by ultrafiltration using a 10 kDa cut-off type YM-10 membrane (Amicon), and was then centrifuged for 10 min at 20,000×*g*. Characterization of the enzyme (formerly referred as MnP with Mn-independent activity) is described in Palma et al. (2000).

Oxidation of pharmaceuticals by VP

Pharmaceutical oxidation was carried out in 100 ml Erlenmeyer flasks sealed with Teflon plugs, with magnetic stirring at room temperature, i.e. 22 ± 2°C. The reaction mixture (50 ml) consisted of diverse pharmaceuticals (2.5 mg l^{-1}) and VP (10–1,000 U l^{-1}), Mn^{2+} (33 μM) and sodium malonate (1–10 mM) at pH 4.5. The reaction started with the continuous addition of H_2O_2 at a rate of 1–10 μM min^{-1} (referred to the reaction mixture) supplied by a peristaltic pump at 20 μl min^{-1}. Samples were withdrawn at regular intervals and used in the subsequent analysis to determine pharmaceutical concentrations and the evolution of VP activity. Duplicates of each condition were carried out. To verify that degradation took place only due to enzymatic oxidation, controls were run in parallel in absence of VP but with the continuous addition of hydrogen peroxide.

Quantification of pharmaceuticals by HPLC

The compounds were analyzed quantitatively by high-performance liquid chromatography (HP 1090 HPLC) equipped with a diode array detector, a reverse-phase column: LiChrospher 100 RP-18 (4 mm × 250 mm, Merck) and a HP ChemStation data processor. The operation conditions for the analysis of SMX, CTL and FLX were: 100 μl injection volume, λ 226 nm, eluent acetonitrile: sodium phosphate (50 mM, pH

2.2) in isocratic conditions of 40:60% and at a flow rate of 0.9 ml min^{-1}. Retention times were 7, 11 and 19 min for SMX, CTL and FLX, respectively. Conditions for the analysis of CBZ, DCF and NPX were: 200 µl injection volume, λ 220 nm, eluent acetonitrile:phosphate (50 mM, pH 4.5) in isocratic conditions of 45:55% at a flow rate of 0.8 ml min^{-1}. Retention times were 7, 10 and 16 min for CBZ, NPX and DCF, respectively. Conditions for the analysis of E1, E2 and EE2 were: 200 µl injection volume, λ 210 and 275 nm, acetonitrile:water as eluent starting at 50:50 and ending at 90:10% after 25 min, at a flow rate of 0.8 ml min^{-1}. The retention time for E2 was 14 min and for E1 and EE2 was 16 min. Since E1 and EE2 eluted at the same retention time, E1 degradation experiments were run independently. Detection limits for all the pharmaceuticals and estrogens were 0.1 mg l^{-1}.

Identification of sulfamethoxazole degradation products

Experiments with an initial concentration of 25 mg l^{-1} of SMX, 200 U l^{-1} VP, 33 µM Mn^{2+}, and 1 mM sodium malonate were run in order to identify SMX degradation products. Acidified samples at time 0 and after 12 h of enzymatic treatment were analyzed by liquid chromatography electrospray time-of-flight mass spectrometry (LC–ESI–TOF–MS) in positive ionization mode. A HPLC (Agilent 1100) equipped with a Zorbax Eclipse XDB (C18 3 mm × 250 mm, 5 µm, Agilent) analytical column was used. Mobile phases were acetonitrile plus 0.1% formic acid (A) and water with 0.1% formic acid (B), respectively, at a flow rate of 0.4 ml min^{-1}. A linear gradient progressed from 10% A (initial conditions) to 100% A in 50 min, and maintained at 100% A for 3 min. The injection volume was 20 µl and oven temperature at 25°C. Under these conditions, SMX retention time was 18 min. This HPLC system was connected to a Microtof Bruker Daltonics mass spectrometer with an electrospray interface operating under the following conditions: capillary, 4500 V; nebulizer, 2.5 bar; drying gas, 8.0 l min^{-1}; gas temperature, 180°C. Ion chromatography (Metrohm 861) was carried out to determine anion concentrations (nitrate and nitrite) and carboxylic acids, using a Metrosep A supp 5 column (250 mm × 4.0 mm, Metrohm) at the conditions specified by the supplier.

Enzymatic activity assay

Versatile Peroxidase activity was determined spectrophotometrically (Cecil CE 7200) at 30°C and 468 nm. The method is based on the oxidation of 2,6-dimethoxyphenol (2,6-DMP) by VP system: extinction coefficient 49,600 M^{-1} cm^{-1} (Wariishi et al. 1992). VP assays were carried out with 200 µl sodium malonate (250 mM, pH 4.5), 50 µl 2,6-DMP (20 mM), 50 µl $MnSO_4$ (20 mM), and the sample (50 µl). The reaction was started by the addition of 100 µl H_2O_2 (4 mM), ending up with a volume of 1 ml. One unit of activity was considered as the amount of enzyme which forms one µmol of product per min.

Results

Degradation of sulfamethoxazole

In this study, the kinetics of SMX degradation by VP was evaluated. The effect of the malonate concentration (1–10 mM) and hydrogen peroxide addition rate (1–10 µM H_2O_2 min^{-1}) was analyzed. This range was selected based on previous experiments for degradation of recalcitrant compounds, such as polycyclic aromatic hydrocarbons and dyes (Mielgo et al. 2003; Eibes et al. 2006). Assuming first-order kinetics, the constants were determined by plotting logarithmic concentrations against time. The experimental conditions evaluated and the results of the degradation of SMX are summarized in Table 2.

The optimization of hydrogen peroxide addition is crucial for the adequate performance of the VP treatment system. Excessively high addition rates can lead to a rapid inactivation of the enzyme, as occurred for 10 mM malonate and 10 µM H_2O_2 min^{-1} where two-thirds of the initial activity was lost in 2 h. On the contrary, a low concentration of H_2O_2 may limit the action of VP, being insufficient for the enzyme to effectively complete the catalytic cycle.

Increasing the concentration of sodium malonate had a negative effect on the degradation kinetics when 1 or 5 µM H_2O_2 min^{-1} was added, although it did not cause any significant effect on the inactivation of the enzyme. The highest kinetic constant, 0.25 h^{-1} ($r^2 = 0.94$), was achieved working at 1 mM malonate and 5 μM H_2O_2 min^{-1}. The profile of SMX

Table 2 Degradation results for pharmaceuticals with different malonate-H_2O_2-enzyme ratios

Compound	Malonate (mM):H_2O_2 ($\mu M\ min^{-1}$)	VP ($U\ l^{-1}$)		Time	Degradation	k	r^2
		Initial	Final	(h)	(%)	(h^{-1})[a]	
SMX	1:1	195	85	7	77	0.24	0.96
	1:5	210	40	7	80	0.25	0.94
	1:10	150	25	7	64	0.16	0.94
	10:1	175	70	7	76	0.20	0.94
	10:5	180	45	7	76	0.19	0.93
	10:10	190	35	7	77	0.17	0.87
CTL	1:5	990	195	7	18	0.03	0.77
FLX	1:5	990	195	7	<10	–	–
NPX	1:5	245	55	7	55	0.12	0.88
		1110	330	7	80	0.21	0.86
DCF	1:1	10	2	0.42	100	9.00	0.99
CBZ	1:5	1110	330	–	–	–	–
EE2	1:5	55	30	0.17	100	46.0	0.99
		10	7	0.17	100	30.6	0.99
	1:1	50	30	0.25	100	17.4	0.94
		10	6	0.25	100	13.0	0.96
E2	1:5	55	30	0.08	100	–[b]	–
		9	7	0.17	100	35.9	0.99
	1:1	50	30	0.25	100	20.7	0.93
		10	6	0.25	100	14.8	0.97
E1	1:1	10	4	0.17	100	19.3	0.94

[a] The degradation profile was adjusted to first order kinetics

[b] Kinetic coefficient not determined because complete degradation took place in less than 5 min

concentration and the control (in absence of enzyme but with the addition of H_2O_2) throughout time under those optimal conditions are shown in Fig. 1a. The overall degradation of SMX was around 80% after 7 h, with an average degradation rate of 0.24 mg l^{-1} h^{-1}. SMX was mainly degraded during the first 4 h (70%) and when activity decreased below 100 U l^{-1}, the elimination of the contaminant slowed down.

Products from the enzymatic degradation were determined by LC-TOF–MS and by ion chromatography. Although the formation of several compounds was observed by LC–MS, only one compound, which eluted at 6.2 min, was identified as 3-amino-5-methylisoxazole (*m/z* 99.0558, protonated molecule) and confirmed by comparison with an authentic standard. A larger molecular weight (*m/z* 503.3166, protonated molecule) observed at a retention time (RT) of 25.9 min, may correspond to a product from the dimerization of SMX (Dodd and Huang 2004). Other unidentified intermediates were observed, such as the compound eluted at 27.2 min (*m/z* 158.0270) and the compound eluted at 30.9 min (*m/z* 245.0772). Ion chromatography showed the formation of traces of carboxylic acids such as acetic and oxalic acid, eluted at 7 and 34 min, respectively. Furthermore, anions such as nitrate (RT 19 min), nitrite (RT 13 min) and sulfate (RT 30 min) were produced during the enzymatic transformation, at very low concentrations. The compounds were identified by comparison with authentic standards.

Degradation of pharmaceuticals

The degradation of the antidepressants CTL and FLX was evaluated under the optimal conditions for SMX degradation: 200 U l^{-1} VP, 1 mM malonate and 5 μM H_2O_2 min^{-1} (Table 2). Nevertheless, no significant removal was observed after 8 h of treatment (data not shown). In order to overcome a potential constraint of enzyme concentration, new experiments were performed with an initial VP activity fivefold higher than that of the previous experiments ($\approx$1,000 U l^{-1}). Even with non-limiting conditions of VP (the activity remained above 100 U l^{-1} throughout the experiment),

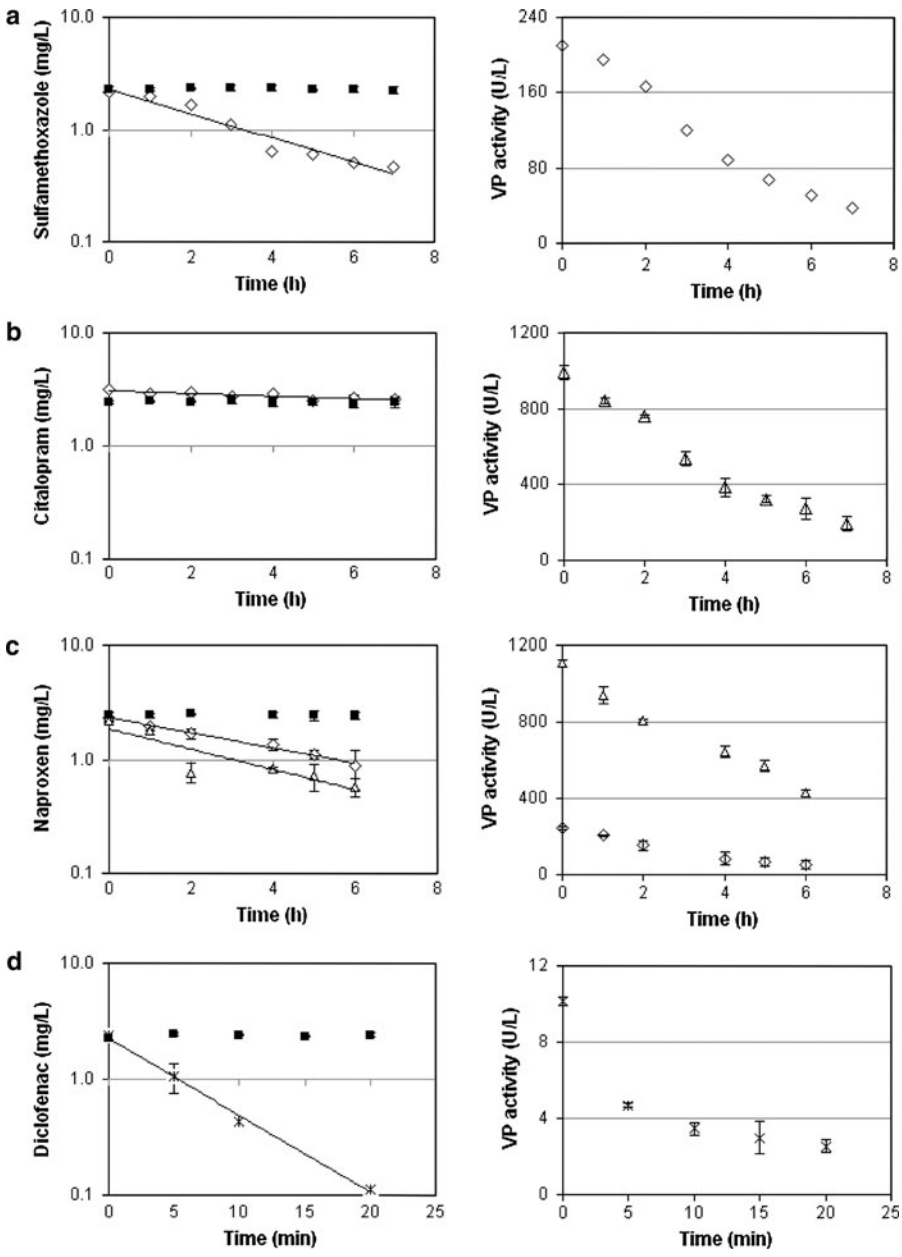


Fig. 1 Concentration of pharmaceuticals (*first column*) and profile of VP activity (*second column*) during enzymatic treatment. *Symbols* Pharmaceutical concentration in control (*filled square*) and assays with 1,000 U l^{-1} VP-5 μM H_2O_2 min^{-1} (*open triangle*), 200 U l^{-1} VP-5 μM H_2O_2 min^{-1} (*open diamond*) or 10 U l^{-1} VP-1 μM H_2O_2 min^{-1} (*asterisk*). *Lines* represent fitting of degradation profiles to first-order kinetics (Table 2). *Bars* represent standard deviation of two independent experiments

the degradation achieved was partial for CTL, 18% after 7 h of enzymatic treatment (Fig. 1b) and lower than 10% for fluoxetine (data not shown). The pseudo first-order kinetic coefficient (k) for CTL degradation was 0.03 h^{-1} ($r^2 = 0.77$) and the average degradation rate was 0.08 mg l^{-1} h^{-1}.

The enzymatic oxidation of NPX and DCF (anti-inflammatories) was conducted under optimal experimental conditions defined in previous experiments: 1 mM malonate, 5 μM H_2O_2 min^{-1} and VP activity ranging from 10 to 1,000 U l^{-1} (Table 2). Both substances had a different behaviour and the initial concentration of enzyme was a critical point related to the degradation extent. NPX was degraded up to 55% ($k = 0.12$ h^{-1}, $r^2 = 0.88$) when starting with ≈200 U l^{-1} of VP (Fig. 1c). Although VP activity decreased to values below 100 U l^{-1} after 3 h of treatment, NPX elimination continued progressively.

A higher initial activity ($\approx$1,000 U l^{-1}) led to the removal of 80% of NPX in 7 h ($k = 0.21$ h^{-1}, $r^2 = 0.86$). The average degradation rates were 0.19 mg l^{-1} h^{-1} and 0.25 mg l^{-1} h^{-1} for the lowest and the highest enzymatic activity, respectively. Regarding DCF, total removal of this compound was immediately detected when initial VP activity was 200 U l^{-1}. Therefore, further research was performed to demonstrate the potential degradation of this compound, even at very low levels of VP. The assay performed with only 10 U l^{-1} VP and 1 μM H_2O_2 min^{-1} showed total degradation after only 25 min (Fig. 1d), corresponding to a kinetic constant of 9.0 h^{-1} ($r^2 = 0.99$) and an average degradation rate of 5.72 mg l^{-1} h^{-1}.

Finally, the degradation of CBZ (antiepileptic) was assessed starting with different enzyme concentrations. No significant removal after 7 h was detected, even using an initial enzyme activity of 1,000 U l^{-1} (data not shown).

Degradation of estrogens

The enzymatic oxidations of the natural estrogens, E1 and E2 and the synthetic product EE2 were evaluated. Different experimental conditions were analyzed for E2 and EE2: (i) VP activity between 10 and 50 U l^{-1} and (ii) H_2O_2 ranging from 1 to 5 μM min^{-1}. The concentration of malonate remained constant in all experiments, with a value of 1 mM. A summary of the conditions evaluated and the results of kinetic coefficients is shown in Table 2. Figure 2 shows the degradation profiles and the controls (in absence of enzyme but with the addition of H_2O_2) for the different conditions. In all tests complete degradation

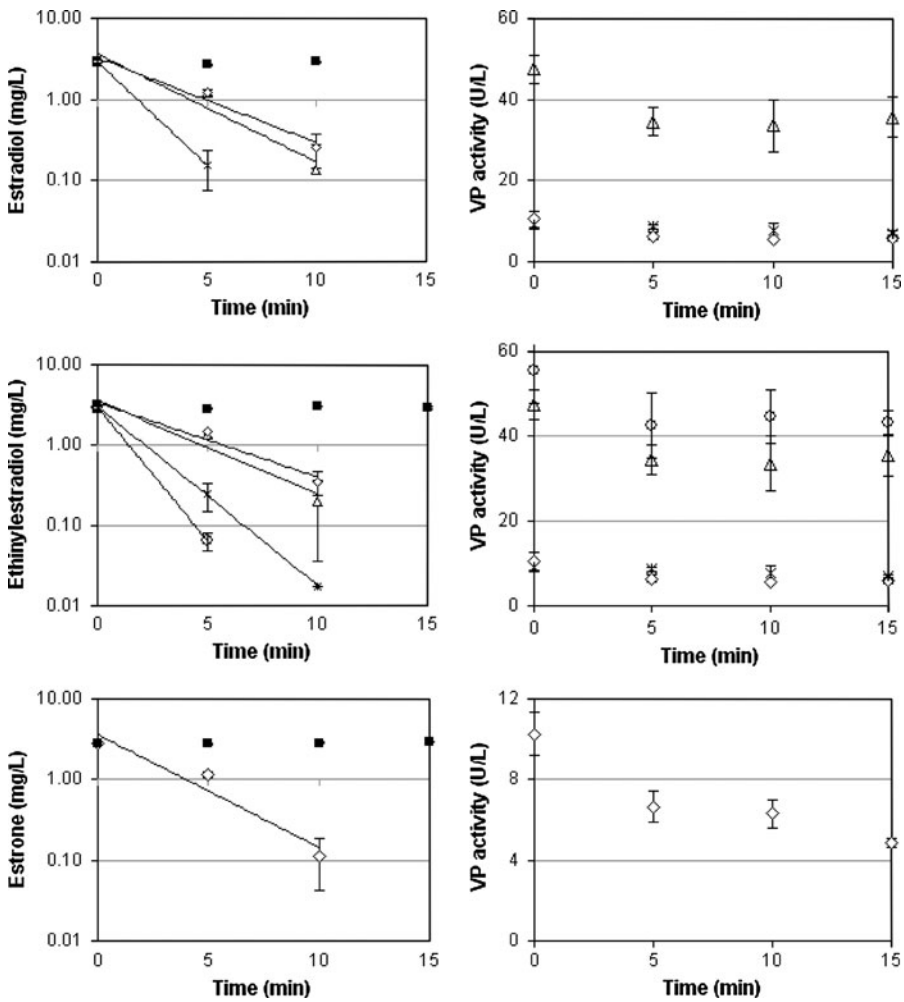


Fig. 2 Concentration of estrogens (*first column*) and profile of VP activity (*second column*) during enzymatic treatment.*Symbols* substance concentration in control (*filled square*) and assays with 10 U l^{-1} VP-1 μM H_2O_2 min^{-1} (*open diamond*), 50 U l^{-1} VP-1 μM H_2O_2 min^{-1} (*open triangle*); 10 U l^{-1} VP-5 μM H_2O_2 min^{-1} (*asterisk*); 50 U l^{-1} VP-5 μM H_2O_2 min^{-1} (*open circle*). *Lines* represent fitting of degradation profiles to first-order kinetics (Table 2). *Bars* represent standard deviation of two independent experiments

of the three compounds was obtained in 15 min or less, depending on the assayed conditions. Average degradation rates ranged from 12 mg l^{-1} h^{-1} (when 1 μM H_2O_2 min^{-1} was used) to 35 mg l^{-1} h^{-1} (E2 with 55 U l^{-1} VP and 5 μM H_2O_2 min^{-1}). Both variables, H_2O_2 and VP concentration, had a positive effect on the oxidation in the evaluated range. Increasing their concentration led to the highest kinetic coefficients. In this way, almost complete degradation would be probably achieved immediately by using higher VP concentrations. Nevertheless, this system can be considered highly efficient, since almost complete degradation of all estrogens was obtained after 5–10 min under minimal requirement conditions.

Discussion

The efficiency of the enzymatic treatment was evaluated by analyzing the removal efficiency in comparison with other types of conventional treatments or advanced oxidation processes.

Antibiotics: sulfamethoxazole

Prolonged time periods are required for the biological removal of SMX at WWTPs (Carballa et al. 2004; Drillia et al. 2005); hence, different advanced oxidation processes (AOPs) have been proposed as alternatives (Ikehata et al. 2006). Ozonation has been proved to be a suitable method to remove SMX in water (Dantas et al. 2008). Nevertheless, a high ozone dosage would be necessary to achieve the complete mineralization of the intermediates. To the best of our knowledge, this is the first study where SMX was demonstrated to be degraded by a ligninolytic peroxidase. The successful results, according to the obtained degradation percentage and the duration of the enzymatic treatment, indicated that VP treatment could be considered as an alternative for the degradation of the antibiotic. The rate of addition of hydrogen peroxide was a key parameter in the degradation kinetics. It must be strictly controlled, since an excessive fast addition rate would cause the pathway of the oxidation process to be diverted towards the partial inactivation of the enzyme. On the contrary, an excessively slow addition rate may limit the reaction rate and extent. In order to provide an economically viable technology, strategies such as immobilization or addition of stabilizers may be studied.

Since amino groups are known substrates of VP enzyme (Hofrichter 2002), SMX is likely to be degraded in two different sites: the amine aromatic site and the amine in the central structure. In fact, the identification of the degradation product AMI evidenced the attack of SMX at the amine site in the central structure. Ionic chromatography showed the formation of carboxylic acids such as oxalic and acetic acid. Furthermore, anions such as nitrate, nitrite and sulfate were produced during the enzymatic transformation. Those compounds have been previously reported as intermediates during the removal of aqueous SMX by different advanced oxidation processes such as photo-Fenton (Trovo et al. 2009), titanium dioxide photocatalysis (Hu et al. 2007), chlorination (Dodd and Huang 2004), etc.

Antidepressants (citalopram and fluoxetine)

CTL and FLX are highly persistent to chemical and biological treatment. For example, Kwon and Armbrust (2005, 2006) studied the photodegradation of these compounds, finding a partial degradation under alkaline conditions (pH 9.0) with a half-life of 64 days. Recently, Redshaw et al. (2008) investigated the biodegradability of FLX in liquid cultures (60 days) and in soils (>200 days), and found these pharmaceuticals to be highly persistent. Such recalcitrant behaviour has also become evident for the enzymatic oxidation of FLX, since no significant degradation was detected after 7 h of treatment. On the other hand, data related to the biodegradation of CTL is still scarce, and no references for enzymatic treatment are found. In the present work, there is evidence of enzymatic oxidation of CTL, but it is limited. Thus, the enzymatic system needs further research to optimize the process in order to consider it a feasible alternative for the treatment of water effluents.

Anti-inflammatories (naproxen and diclofenac)

NPX has been degraded by VP at a removal rate similar to that of conventional biological systems present at WWTPs (Carballa et al. 2004). However, the high demand of enzyme limits its applicability and makes imperative the optimization of the process.

An in vivo system using *Trametes versicolor* attained total degradation after 6 h (Marco-Urrea et al. 2010a). The authors suggested that both the ligninolytic enzyme laccase and the intracellular enzyme cytochrome P-450 played a role in NPX degradation. Other alternatives to enzymatic/fungal treatment could be based on the use of ozone as a processing technique, but a high O_3 concentration (15 mg l^{-1}) is also necessary for a complete removal of NPX (Gagnon et al. 2008).

Degradability of DCF is low in the aerobic biological treatment (up to 30%) and highly variable in the anaerobic treatment (between 0 and 75%) (Carballa et al. 2004). Ozone can degrade DCF, but the results are largely dependent of the quality of the upstream effluent (prior to the ozone treatment step). Ikehata et al. (2006) described complete degradation by several AOPs in high quality wastewater. Gagnon et al. (2008) reported an elimination of 80% (with O_3 concentration ranged from 15 to 30 mg l^{-1}) for primary-treated wastewater. Removal of DCF by the white rot fungus *Phanerochaete sordida* in a 6-day period has also been demonstrated, reporting the removal of the acute lethal toxicity (Hata et al. 2010a). Alternatively, only 24 h were sufficient to completely remove DCF and intermediates by *T. versicolor* pellets (Marco-Urrea et al. 2010b). In view of the results presented here, enzymatic degradation could be studied as a potential alternative for WWTP, since a total removal of DCF in short times (25 min) and at very low enzyme concentration was observed. Another ligninolytic enzyme, LiP, was used to remove DCF, obtaining complete degradation of 5 mg l^{-1} in 2 h using considerably higher amounts of H_2O_2 (3 mg l^{-1}) (Zhang and Geißen 2010). However, the concentration of enzyme, which is likely to determine the feasibility of the process, was not optimized.

Antiepilectics (carbamazepine)

CBZ has been proven to be very recalcitrant since it by-passes sewage treatment (Carballa et al. 2004). High reactivity of CBZ towards ozonation and other AOPs were reported by several groups of researchers, with complete degradation and up to 30% mineralization (Ikehata et al. 2006). Recently, Marco-Urrea et al. (2009) have reported that different ligninolytic fungi were able to degrade CBZ (up to 50%) after a 7-day incubation period. The authors suggest that the intracellular degradation occurs via the cytochrome P-450 system and extracellular fungal enzyme system did not appear to play a role in the first step of degradation, confirming our observations. A single treatment with laccase-HBT was partially effective removing CBZ, but a repeated treatment eliminated 60% after 48 h (Hata et al. 2010b). Besides, Zhang and Geißen (2010) found that CBZ was hardly degraded by LiP (mostly below 10%). For future research, lipid peroxidation may be considered (Bogan et al. 1996). This process occurs when unsaturated lipids are present, generating powerful oxidative radicals which help to decompose those recalcitrant compounds not directly oxidized by VP.

Estrogen hormones

So far, several research groups reported the effective decomposition of E1, E2 and EE2 by chemical and biological treatments (Carballa et al. 2004; Huber et al. 2004; Ikehata et al. 2006). Estrogenicity associated with these compounds can be reduced by different AOPs such as O_3/H_2O_2, H_2O_2/UV and TiO_2/hν processes to virtually undetectable levels (Rosenfeldt and Linden 2004). Ligninolytic enzymes have been demonstrated to be able to remove estrogens. Suzuki et al. (2003) and Tamagawa et al. (2006) reported the degradation of E1, E2 and EE2 by MnP or laccase following 1 h treatment. Interestingly, the authors observed that estrogenic activity was completely removed in a period of 2–8 h of enzymatic treatment. In the present work, we propose a similar system for the in vitro elimination of estrogens, but simpler and more efficient with an enzyme activity 60-fold lower.

Conclusions

The potential degradation of a selection of six pharmaceuticals and three estrogens by VP from *B. adusta* was studied. The results obtained show that the oxidation of antibiotics (SMX), anti-inflammatories (DCF and NPX) and hormones (E1, E2 and EE2) may be significant, reaching high degradation rates even at low enzymatic requirements. On the contrary, antidepressants (CTL and FLX) and the antiepileptic (CBZ) are much more recalcitrant and further optimization of the in vitro technology may be required.

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