Bioconversion of Non-steroidal Anti-inflammatory Drugs Diclofenac and Naproxen by Chloroperoxidase

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Abstract

Non-steroidal anti-inflammatory drugs diclofenac and naproxen are widely used for the treatment of arthritis, ankylosing spondylitis, and acute muscle pain. However, most of them are usually not metabolized and simply pass through human body. These drugs are difficult to be decomposed by general waste treatment strategies and have caused serious environmental concerns. We report a rapid and efficient conversion of diclofenac and naproxen by chloroperoxidase-catalyzed H_2O_2 -oxidation, a heme protein isolated from *Caldariumyces fumago*. Complete conversion of diclofenac and naproxen was achieved in only 9 and 7 min respectively with $0.1 \text{ mmol} \cdot \text{L}^{-1} \text{H}_2\text{O}_2$ and nanomolar enzyme concentration at pH 3.0. The converted products were identified by HPLC-MS and NMR, suggesting involvement of multiple steps in CPO catalyzed conversion. Our work demonstrated that CPO treatment (with COD removal of 4.9% , 9.1% for diclofenac and naproxen, respectively) followed by existing bioremediation technologies (activated sludge) greatly improved the decontaminating these two drugs from waste water (COD removal was enhanced to 85% and 86%, respectively). The eco-toxicity evaluation according to the 72-h EC_{50} value using the green algae *Chlorella Pyrenoidos* as ecological indicators showed that the converted products of diclofenac and naproxen had lower toxicity than the original drugs.

Keywords: enzymatic conversion; diclofenac; naproxen; chloroperoxidase; eco-toxicity

1. Introduction

Both diclofenac (2-[(2,6-dichlorophenyl)amino]benzeneacetic acid; sodium salt) and naproxen (2-(6-methoxynaphthalen-2-yl)propionic acid) (Fig. 1) are non-steroidal anti-inflammatory drugs (NSAIDs). They are widely used in the treatment of arthritis, ankylosing spondylitis, acute muscle pain, and other symptoms associated with inflammation. The widespread application of these drugs coupled with their resistance toward metabolic breakdown in human body inevitably resulted in the release of large amount of these drugs into the aquatic environment. In recent years, diclofenac and naproxen has been detected in surface water, groundwater, wastewater, and even in drinking water at concentrations ranging from $\text{ng}\cdot\text{L}^{-1}$ to $\mu\text{g}\cdot\text{L}^{-1}$ [1,2]. It has been reported that naproxen has adverse effects on biota, such as impairing the lipid peroxidation system of bivalves and altering microbial community structure to yield antibiotic resistance in environmental microbial communities [3]. Diclofenac was recently designated as an environmental pollutant because of its bioaccumulation in the food chain [4]. Therefore, effective conversion of these drugs is of great significance for reducing their accumulation in the environment.

Unfortunately, conventional sewage treatment is inefficient in removing or converting diclofenac and naproxen. For example, no conversion of diclofenac was observed over a period of 28 days either when it was the sole source of carbon itself or when an external carbon source was added for co-metabolic conversion [5]. Batch tests using an activated sludge system and membrane bioreactors also revealed very poor conversion of diclofenac compared with other pharmaceuticals [6]. Besides, physicochemical approaches including ultrasonic [7], electrochemical oxidation [8], photocatalytic [9], potassium permanganate

oxidation [10] and ozone oxidation [11] have been used to treat the effluent containing these drugs. However, limitations of these methods, such as cost, efficiency, and secondary pollution from use of chemicals, make further investigations necessary to removal these drugs from environment.

Little evidence of diclofenac transformation by microbial communities is documented in the literature. White-rot fungi (WRF) were found to be efficient in degrading both diclofenac [12] and naproxen [13], but long treatment time (1 h and 5 h, respectively) was needed. Enzymatic conversion is an ideal approach because of the mild and environment-friendly reaction conditions. Crude lignin peroxidase (LiP, a heme-imidazole peroxidase) from *Phanerochaete chrysosporium* has been used to convert diclofenac in various conditions and was found to be highly effective [14]. In addition, fungal unspecific peroxygenases (UPOs) have been shown to be able to hydroxylate/O-demethylate a diverse array of pharmaceuticals (including diclofenac and naproxen) [15-17].

In this work, inspired by the results of LiP and UPOs, we studied the conversion of diclofenac and naproxen by chloroperoxidase (CPO), a heme containing glycoprotein that is functionally (haloperoxidases) related to UPOs with higher oxidative potential and thermal/chemical stability than LiP [18,19]. High performance liquid chromatography-mass spectrometry (HPLC-MS) and nuclear magnetic resonance (NMR) spectroscopy were used to identify the major conversion products, and the reaction pathways are postulated accordingly. Moreover, the eco-toxicity of the converted solution was evaluated based on EC_{50} value (drugs concentration required to cause 50 % reduction in growth) on the growth of freshwater green algae, *Chlorella pyrenoidosa*. This is one of our serial studies on the degradation of

contamination in aqueous solution, including pesticide and dyes *etc* by this versatile enzyme [20,21].

2. Experimental

2.1 Enzyme preparation

Chloroperoxidase was isolated from the growth medium of *C. fumago* (obtained from Guangdong province microbial culture collection center) according to the method of Morris and Hager [22] with minor modifications, using acetone rather than ethanol in the solvent fractionation step. CPO with $R_z = 1.03$ (A_{398}/A_{280} , 1.44 for pure enzyme) was prepared and stored in 100 mM phosphate buffer (pH 5.0) at 4 °C. The halogenation activity of CPO used in this study was $4232 \text{ U} \cdot \text{mL}^{-1}$ based on the standard monochlorodimedon (MCD) assay [23]. The aromatic hydroxylation activity ($3563 \text{ U} \cdot \text{mL}^{-1}$) of the enzyme was determined by monitoring the hydroxylation of naphthalene into 1-naphthol [24]. The classic peroxidase activity of the enzyme determined using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) as substrate was $3071 \text{ U} \cdot \text{mL}^{-1}$ [25].

2.2 Reagents and buffers

All reagents used in this study, including diclofenac, naproxen, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, hydrogen peroxide (30 % in aqueous solution), ethyl acetate, and inorganic reagents for cultivating the green alga were obtained from Xi'an Chemical Co. Ltd (Xi'an, China) with highest purity ($\geq 98\%$). Other chemicals such as methanol and acetonitrile (chromatography grade) as well as standard conversion product of the two drugs, o-desmethylnaproxen and 4'-hydroxydiclofenac (chromatography grade) were purchased from Sigma Aldrich (St. Louis, MO USA).

Phosphate buffer (0.1 mol·L⁻¹) was prepared by mixing appropriate volumes of 1 mol·L⁻¹ KH₂PO₄ and K₂HPO₄ stock solutions and diluting the combined solutions to 1 L. Then the solution was adjusted to various pH with 1 mol·L⁻¹ H₃PO₄. All solutions were prepared using deionized water with a conductivity of 5.6×10⁻⁸ s·cm⁻¹.

2.3 Conversion of diclofenac and naproxen

In a typical experiment, enzymatic conversion of both drugs was carried out in 0.1 mol·L⁻¹ phosphate buffer in a centrifugal tube with the total volume of 3.0 mL containing CPO (5.0 nmol·L⁻¹ for diclofenac, and 20.0 nmol·L⁻¹ for naproxen), 20 mmol·L⁻¹ KCl, and drugs (15 μmol·L⁻¹) at pH 3.0 at 298K. The reaction was started by adding H₂O₂ (0.1 mmol·L⁻¹) with magnetic stirring and continued for 20 min. The reaction conditions were optimized according to single factor investigation, with pH at range of 2~6, CPO concentration at 0.25-23 nmol·L⁻¹, H₂O₂ concentration at 0.015~0.3 mmol·L⁻¹, and reaction time of 0~20 min. The effect of each factor on conversion efficiency was investigated when the other reaction conditions were kept at optimum points as in the above typical experiment. The supernatant of the reaction mixture was extracted 3 times using ethyl acetate. The combined organic extract was concentrated by rotary evaporation (0.09 MPa, 308K) to remove the solvent. The extracts were then dissolved in acetonitrile and methanol, respectively, for HPLC (LC-20AT, Shimadzu) analysis. The mobile phase consisted of 80:20 (v/v) acetonitrile and water for diclofenac and 90:10 (v/v) methanol and water for naproxen. The flow rate was 0.5 ml·min⁻¹. The detector was set at 274 nm and 235 nm for diclofenac and naproxen, respectively. The quantitative analysis of the target compounds was based on the standard curve (correlation coefficients were > 0.999).

$$\text{Conversion efficiency } \eta = ((C_0 - C_t) / C_0) \times 100\% \quad (1)$$

Where C_0 and C_t were the concentration of the drugs at reaction time 0 and t , respectively.

All sets of experiments were reproduced several times under identical operating conditions to ensure the accuracy, and each data was obtained at least three times. The reported data were mean values of three independent measurements with the discrepancy was below 5%.

2.4 Determination of products

Converted solution was obtained at the optimum reaction condition with $0.1 \text{ mmol} \cdot \text{L}^{-1} \text{H}_2\text{O}_2$, CPO ($5.0 \text{ nmol} \cdot \text{L}^{-1}$ for diclofenac, and $20.0 \text{ nmol} \cdot \text{L}^{-1}$ for naproxen) at pH 3.0. After 30min reaction time, samples were collected and treated as above for HPLC-MS analysis. An Esquire LC-ion trap mass spectrometer (Bruker Daltonics, Germany) equipped with an orthogonal geometry Electrospray Ionization (ESI) source was used to determine the formulae of the products. Nitrogen was used as the drying ($8 \text{ L} \cdot \text{min}^{-1}$) and nebulizing (0.8 bar) gas at 180°C . Scanning was from m/z 100 to 1000 in the standard resolution mode.

To determine the structure of the conversion products, the reaction was carried out using the same condition as stated above except a larger volume (300 mL) was used. Upon completion of the reaction, the reaction mixture was extracted with either ethyl acetate or chloroform. After removal of the solvent by rotary evaporation (0.09 MPa, 308K) the extracts were dissolved in either deuterated chloroform or methanol and transferred to 5 mm NMR tubes. NMR experiments were carried out on a Bruker 600 MHz NMR spectrometer, operating at a proton frequency of 599.73 MHz. All spectra were recorded at 298 K using standard pulse programs from the manufacturer.

2.5 Elimination of COD and TOC

Total organic carbon (TOC) measurement was conducted on a TOC-VCPA analyzer

(Shimadzu Corp.). The feed speed was $150 \text{ ml} \cdot \text{min}^{-1}$. Chemical oxygen demand (COD) was measured by a quick method on a Rapid Autoanalyzer (5B-1(F), Lian-hua Tech.Co., Ltd). A solution containing 2.5 ml sample, 0.7 ml reagent D (potassium dichromate) and 4.8 ml reagent E (catalysts) in a 20.0 ml glass tube was heated to 438K and kept for 10 min together with a blank sample and a standard sample. After 2 min of air cooling, the heated solution was cooled by water for another 2 min. Then the absorbency of the samples was measured at 610 nm. The sample for COD measurement was collected after 30min reaction time with $0.1 \text{ mmol} \cdot \text{L}^{-1} \text{H}_2\text{O}_2$, CPO ($5.0 \text{ nmol} \cdot \text{L}^{-1}$ for diclofenac, and $20.0 \text{ nmol} \cdot \text{L}^{-1}$ for naproxen) at pH 3.0.

2.6 Treatment of drug effluent by activated sludge

A mixed population of activated sludge microorganisms was collected from Xi'an second sewage treatment plant (Xi'an, China). The sample of activated sludge was filtered on a Spectra Mesh polypropylene 149- μm filter (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) to remove aggregates. The sample was then washed three times by centrifugation and suspended in the same volume of culture medium. To remove any excessive amounts of dissolved organic carbon, the suspension was stirred and maintained under oxygen at 298K for at least 24 h without exposure to the test materials. The volatile ratio f of activated sludge was 0.74.

90 mL of $15 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$ drug effluent was put into the activated sludge suspension before /after enzymatic treatment (to ensure a final drug concentration of $7.5 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$). The samples were magnetically stirred all the time when air was used as the aerated gas. The sample was then left to stand for 1-2 h. The supernatant was taken for determination of COD.

2.7 Toxicity Tests

Freshwater unicellular green alga, *C. pyrenoidosa* (provided by the Institute of Wuhan Hydrobiology, Chinese Academic of Science), was cultivated in nutrient media of blue green medium (BG11) at 298K and illuminated with cool-white fluorescent lights at a continuous light intensity of 2000 Lx. For cell experiments, *C. Pyrenoidosa* was exposed, during its log growth phase, to the toxicant at five different concentrations (maintain final concentration ranging from 0.01 to 0.36 mg/L) for 3-4 days at 298K. The concentration of the alga was determined by monitoring the change of absorption at 680 nm. The toxicity tests for each drug concentration were conducted in triplicate.

The growth inhibition rate i for each sample was calculated from Equation (2) and (3) [26],

$$\mu = (\ln OD_t - \ln OD_{t_0}) / (t - t_0) \quad (2)$$

$$i = (\mu_c - \mu_t) / \mu_c \times 100\% \quad (3)$$

where $t-t_0$ is incubation time (min); OD_{t_0} is absorbance at t_0 ; OD_t is absorbance at t ; μ is growth rate of all life (min^{-1}); μ_c is growth rates of control culture without toxin addition (min^{-1}); and μ_t is growth rates of cultures in the presence of drugs (min^{-1}). EC_{50} (drug concentration required to cause 50% decrease in growth) values were calculated using linear regression analysis of drug concentration as natural logarithm versus percentage inhibition. All correlation coefficients were > 0.99 .

3. Results and discussion

3.1 Effect of reaction parameters on drug conversion

Since various physicochemical parameters influence the conversion efficiency, it is essential to optimize these factors in order to make the process more efficient and practically applicable.

The pH range investigated was 2-6 due to poor stability and activity of CPO at higher pH. Fig. S1 (A) showed that the conversion efficiency of naproxen and diclofenac increased rapidly with increasing pH, and reached maximum around pH 3.0. Then, the conversion efficiency decreased sharply when pH was increased above 3.0. The preference for such a low reaction pH has been observed for most CPO catalyzed reactions and has been attributed to the protein's unique active site structure and catalytic mechanism [20,27]. CPO uses a glutamic acid (Glu 183) as the acid-base catalyst to aid the formation of compound I, which can be best described as an oxyferryl π -cation radical that can oxidize substrate [28]. The protonation of Glu183 is necessary for both the formation of compound I and the binding of negatively charged substrates to avoid unfavorable electrostatic interactions. Acidic environment is also needed to protonate compound X involved in most CPO catalyzed halide dependent reactions [29].

It is well known that high concentration of H_2O_2 (oxidant) inactivates most heme containing enzymes due to internal oxidative destruction of the porphyrin prosthetic group [30]. Thus low concentrations of H_2O_2 are employed for reactions catalyzed by most heme peroxidases. This strategy cannot be simply applied to CPO because of its ability to disproportionate hydrogen peroxide. Therefore, the optimum concentration of hydrogen peroxide needs to be determined based on the actual substrate. The effect of H_2O_2 concentration on CPO catalyzed conversion of diclofenac and naproxen is shown in Fig. S1(B). As expected for all peroxidases, no conversion of either drug was observed before the addition of H_2O_2 . The conversion efficiency of both drugs increased as H_2O_2 concentration is increased. Maximum rate is achieved when total H_2O_2 concentration reached $0.1 \text{ mmol}\cdot\text{L}^{-1}$. Further addition of

H₂O₂, however, repressed the conversion of both drugs possibly due to formation of compound III caused by high concentrations of H₂O₂ [31]. Therefore, 0.1 mmol·L⁻¹ of H₂O₂ was chosen in the conversion of both diclofenac and naproxen in all subsequent experiments.

Optimizing CPO concentration can not only improves conversion efficiency but also save cost of operation. The range of CPO concentration tested was 0.25-6.0 nmol·L⁻¹ for diclofenac and 1-23.0 nmol·L⁻¹ for naproxen. Fig. S1 (C) showed that conversion efficiency of both drugs increased rapidly as CPO concentration was increased. Complete conversion of the drugs was achieved when CPO concentration was above 5.0 nmol·L⁻¹ for diclofenac, and 20.0 nmol·L⁻¹ for naproxen, indicating that CPO is extremely efficient in the conversion of the subject drugs.

CPO convert both diclofenac and naproxen with a remarkable rate. As shown in Fig. S1 (D), about 70% of diclofenac and 75% of naproxen is converted within 1 min. The complete conversion was achieved in only 9 and 7 min for diclofenac and naproxen, respectively, at optimum reaction condition.

3.2 Determination of products by HPLC-MS and NMR

Tremendous efforts have been made to characterize the intermediates and final products generated from diclofenac and naproxen conversion [8,13]. Plausible mechanisms have been proposed for both drugs based on the intermediates and final products identified from different processes. To understand the mechanisms of CPO catalyzed conversion and to evaluate the potential of this enzymatic method in environmental and industrial applications, both HPLC-MS and NMR analyses are employed to establish the structures of the putative products from the two drugs.

The hydroxylation of aromatic rings and O-demethylation of esters have been reported for several unspecific peroxygenase (UPOs) [15-17], a class of heme-thiolate proteins related to CPO and cytochromes P450. However, these two activities have not been reported for CPO. In this work, CPO catalyzed hydroxylation of diclofenac and O-demethylation of naproxen were observed. The conversion of both diclofenac and naproxen was confirmed by HPLC analysis of the reaction product as shown in Fig. S2. The MS spectra of samples from diclofenac conversion (Fig.2) suggested the formation of six putative products. The peak that eluted at 2.6 min (Fig.2A) shows a molecular ion $[M-H]^-$ at m/z 311, indicating the addition of an oxygen atom to the parent compound. Multiple possibilities exist as oxygen can be added at all possible positions of the two benzene rings. However, NMR analysis (Fig.3) suggested compound [II] as the major product corresponding to this molecular ion. Since the coupling pattern of the chloride bearing ring remained in the product, the oxygen must have been added to the acetate bearing ring with oxygen added at the para-carbon of the amino group. The NOE between peak “a” at 3.77 ppm (singlet, 2H) and peak “b” at 7.25 ppm (Fig.S3), the COSY correlation and splitting pattern confirm the structure of compound [II] as reported previously [32-34]. Compound [I], 4'-hydroxydiclofenac, is commercially available and displays a quite different NMR spectral pattern as shown in Fig. S4 and Fig. S5). The peak eluted at 3.1 min (Fig.2B) displayed a molecular ion $[M-H]^-$ at m/z 328, indicating the addition of two oxygen atoms to the parent compound or the addition of one oxygen atom to compound [II]. This product also showed a major fragmentation ion at m/z 284 (Fig.2B), assignable to the decarboxylation of the major product. Decarboxylation was also observed for diclofenac itself (Fig.S6). The NMR spectrum (Fig.S7) of this product showed the absence

of a three proton coupling pattern in the aromatic region as displayed by both the parent compound and compound [II], suggesting the oxygen atom is added to the chloride bearing ring as shown in compound [IV]. This is in good agreement with observations made with other methods [32,34]. The peak eluted at 2.7 min showed a molecular ion $[M-H]^-$ at m/z 325 (Fig.2C), assignable to compound [V] or [VI] due to further oxidation of product [II] or [IV]. However, neither compound [V] nor [VI] was detected in NMR experiments, implicating that these products might form only in the ionization process within mass spectrometer.

Based on the products identified from our LC-MS and NMR analysis, the sequence of diclofenac conversion catalyzed by CPO is proposed (Fig.4). The diverse catalytic activity of CPO makes it possible to produce a broad array of products from diclofenac conversion, however, under the conditions employed in our study, only hydroxylation activity is observed. This can be appreciated by the similarity between CPO and cytochromes P450 that metabolize most xenobiotics via hydroxylation. Thus CPO converts diclofenac to either monohydroxylated or dihydroxylated products, the same as the major products observed in the metabolism of diclofenac [32]. The close similarity among possible hydroxylation products makes it difficult to isolate and purify individual product. This drawback does not comprise the aim of this study.

The identification of products from CPO catalyzed conversion of naproxen is achieved by detailed NMR analysis of the products with the aid of MS. The most noticeable difference between proton NMR spectra of the products and the parent drug, naproxen, is the absence of the methoxy signal (peak “i” around 3.9 ppm, Fig.S8 and S9) in the products as shown in Fig.5. This suggests the demethylation of naproxen as reported previously [19,35]. Although

CPO catalyzed N-demethylation has been reported [36], the observed O-demethylation represents a novel activity of CPO. This is reminiscent of the activity displayed by P450 [37] and UPOs [15] that are structurally related to CPO. The methyl (peak “a” at 1.53 ppm, doublet, $J=6.56$, 3H) and the methine (peak “b” at 4.99 ppm, quartets, $J=6.56$, 1H) signals from the product are indicative of the propionic acid part of naproxen. These signals are downfield shifted compared to the corresponding signals in the parent drug molecule, consistent with observations reported previously [13,35]. Both the methyl and the methine signals displayed NOE to a peak at 7.42 ppm (peak “h”, doublet, $J=1.18$, 1H) and a peak at 7.48 ppm (signal “c”, doublet of doublet, $J=8.03$ and 1.18, 1H) as shown in Fig.5 (A). The peak at 7.48 ppm is strongly coupled to a peak at 8.09 ppm (resonance “d”, doublet, $J=8.03$, 1H) as shown in Fig.5 (B). The NOE and coupling pattern of this product suggests no modification occurred to the propionic group bearing ring of naproxen during degradation. The remaining aromatic signals, peak “g” at 7.44 ppm (doublet, $J=10.11$, 1H) and peak “f” at 6.44 ppm (doublet, $J=10.18$, 1H) are strongly coupled (Fig.5 (B)), demonstrating their vicinal relationship. Based on the above NMR spectral property, this product is proposed to be 2-(5,6-dihydroxynaphthalen-2-yl)propanoic acid due to modification at 5-C of naproxen. However, the NMR spectra of commercially available desmethylnaproxen (Fig. S10 and Fig. S11) do not support the proposed structure of this degradation product. Furthermore, the MS of this product shows a molecular ion $[M-H]^-$ at m/z 311, also inconsistent with the structure proposed (expected m/z at 231). The difference of a mass of 80 between the expected and the observed mass, suggests the phosphorylation of one of the hydroxyl groups. Esterification of desmethylnaproxen has been reported previously [35]. The high phosphate concentration and

the extremely low pH used in our degradation study make it feasible to phosphorylate 2-(5,6-dihydroxynaphthalen-2-yl)propanoic acid and give product (I) as shown in Fig. 6. Product (I) can be further converted to 2-(5,8-dihydroxy-6-(phosphonooxy)naphthalen-2-yl)propanoic acid via hydroxylation at the 8-C. However, this product is not observed in either NMR or MS experiments. The observation of a molecular ion $[M-H]^-$ at m/z 325 suggests the formation of product (II) as shown in Fig.6.

Based on the major products identified from our study, the reaction sequence of naproxen conversion catalyzed by CPO is proposed (Fig.7). Similar as the conversion of diclofenac, CPO catalyzed conversion of naproxen is achieved primarily via CPO's monooxygenase activity. Thus CPO converts naproxen to either monohydroxylated or dihydroxylated products, similar as the major products observed in bacterial conversion of naproxen[38]. Initially, naproxen was converted to desmethylnaproxen[12,39]. The desmethylnaproxen is then phosphorylated to [(2-(6-(phosphonooxy)naphthalen-2-yl)acetic acid [a], not observed)] in the presence of high concentrations of phosphate and under low pH condition employed. Esterification of desmethylnaproxen has been reported in the literature[35]. Next, [a] was hydroxylated to 2-(5-hydroxy-6-(phosphonooxy)naphthalen-2-yl)propanoic acid [I] corresponding to m/z 311 observed in Fig. 6. Product (I) was then converted to 2-(5,8-dihydroxy-6-(phosphonooxy)naphthalen-2-yl)propanoic acid [b, expected $m/z=327$] which is instantly oxidized to 2-(5,8-dioxo-6-(phosphonooxy)-5,8-dihydronaphthalen-2-yl)propanoic acid (II, $m/z=325$) as shown in Fig.6.

The products identified in our NMR and MS analysis are subjected to further oxidative transformation as supported by the results from activated sludge experiment as well as eco-toxicity test.

3.3 Reduction in chemical oxygen demand (COD) and total organic carbon (TOC)

Table 1 showed that only 4.9% , 9.1% of COD and 25%, 7.6% of TOC removal was achieved for diclofenac and naproxen, respectively **after 30min conversion by CPO-H₂O₂**. It is therefore proposed that if CPO catalyzed conversion can serve as an efficient pre-treatment step in waste water treatment following by subsequent bioremediation technologies (activated sludge) for complete decontamination of the two drugs in waste water.

3.4 Combined treatment of drug effluent by enzymatic oxidation and activated sludge

As indicated in Table 1, the COD value of drug effluent did not decrease noticeably after CPO-treatment. However, compared with the parent drugs, the products from CPO catalyzed conversion have improved solubility in aqueous media and are more vulnerable to further biodegradation. Treatment of CPO-catalyzed reaction mixture with activated sludge **after 2h incubation time** increased COD removal from 4.9% and 9.1% to 85% and 86% for diclofenac and naproxen, respectively. The treatment by activated sludge alone **after 12h incubation** only removes 49% and 54% of the COD for diclofenac and naproxen, respectively, suggesting that more effective decontamination of the two drugs can be achieved through CPO pre-treatment followed by existing bioremediation technologies (activated sludge).

3.5 Evaluation of the eco-toxicity of the products

In some cases simple destruction of a drug is inadequate, since the resulting products may also be highly toxic, and special attention must therefore be paid to toxicity assessment of

products to ensure that the agent has been effectively detoxified. However, toxicity evaluation about the products from diclofenac and naproxen are not readily available. Biological assays offer a direct measure to evaluate the magnitude of the potential health risk of chemicals. Therefore, a growth-inhibitory test was carried out using *C. Pyrenoidosa*.

In this work, biological monitoring based on a growth-inhibitory test (72-h inhibition test) was carried out using green algae *Chlorella Pyrenoidosa* as ecological indicators in the toxicity tests because of its high sensitivity to the test solution. The results in Fig. S12 showed an increase of 72-h EC_{50} value with the increasing of conversion efficiency of diclofenac (A) and naproxen (B). EC_{50} increased from 0.14 to 0.29 $\text{mg}\cdot\text{L}^{-1}$ of diclofenac and 0.21 to 0.33 $\text{mg}\cdot\text{L}^{-1}$ of naproxen respectively when the conversion efficiency was 100%. These results demonstrated that the converted products of diclofenac and naproxen had lower toxicity compared with the parent compounds, which can ensure the security of this enzymatic treatment by CPO- H_2O_2 .

4. Conclusion

In summary, our study demonstrated that CPO catalyzed oxidative conversion is a promising alternative for treatment of waste water containing non-steroidal anti-inflammatory drugs. Complete conversion of diclofenac and naproxen is reached in only 9 and 7 min, respectively, under mild conditions.

The products identified by HPLC-MS and NMR suggested the initial hydroxylation of the drug molecules followed by further oxidative transformation.

Though the low COD removal achieved, the biodegradability of the decomposition products was significantly increased. So, the developed CPO- H_2O_2 method can be used for

the pretreatment of wastewater followed by activated sludge treatment for decontamination of diclofenac and naproxen effectively. The products of both diclofenac and naproxen had substantially lower toxicity than the original drugs as judged by the 72-h EC_{50} value of *C. Pyrenoidos*.

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Abbreviations

NSAID, non-steroidal anti-inflammatory drugs; WRF, white-rot fungus; LiP, lignin peroxidase; CPO, chloroperoxidase; HPLC-MS, high performance liquid chromatography-mass spectrometry; NMR, nuclear magnetic resonance; NOE, nuclear overhauser effect; COSY, (homonuclear chemical shift) correlation spectroscopy; MCD, monochlorodimedon; ESI, electrospray ionization; TOC, total organic carbon; COD, chemical oxygen demand; BG, blue green medium; EC_{50} , median effective concentrations;

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Figure captions:

Fig. 1 Chemical structures of diclofenac (a) and naproxen (b)

Fig. 2 ESI-ion trap mass spectra of the conversion products from CPO catalyzed oxidation of diclofenac recorded in negative mode. **A:** the peak (m/z) at 311 and the corresponding supposed products I/II; **B:** the peak (m/z) at 327 and the corresponding supposed products III/IV; the peak (m/z) at 325 and its corresponding supposed products V/VI.

Fig. 3 COSY of diclofenac compound [II] in chloroform-d.

Fig. 4 Proposed conversion pathway of diclofenac during CPO-catalytic oxidative process.

Fig. 5 Proton NMR (A) NOESY and (B) COSY spectra of naproxen product [I]. The weak NOEs between peaks “a” and “c” and between peaks “b” and “c” can only be observed at lower contour levels. The NOE and coupling patterns unequivocally define the structure of this conversion product.

Fig. 6 ESI-ion trap mass spectra of the conversion products from CPO catalyzed oxidation of naproxen recorded in negative mode.

Fig. 7 Proposed conversion pathway of naproxen during CPO-catalytic oxidative process.

Table 1 Elimination of COD and TOC by CPO catalyzed oxidative conversion.

a

b

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Table 1 Elimination of COD and TOC by CPO catalyzed oxidative conversion

substance	COD (mg · L ⁻¹)	COD removal (%)	TOC (mg · L ⁻¹)	TOC removal (%)
diclofenac	41→39	4.9	8.3→6.2	25
naproxen	22→20	9.1	158→146	7.6

graphic Table of Contents

Chloroperoxidase efficiently degrades diclofenac and naproxen under mild conditions.

Complete degradation is in 9 min and 7 min respectively using 10^{-9} mol·L⁻¹ enzyme.