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Toward the development of a biocatalytic system for oxidation of *p*-xylene to terephthalic acid: oxidation of 1,4-benzenedimethanol

John A. Morgan[1], Zhongqing Lu[2], Douglas S. Clark*

Department of Chemical Engineering, University of California, Berkeley, CA 94720, USA

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Abstract

The oxidation of hydrocarbons is an important value-enhancing chemical transformation. Current inorganic catalysts often operate under harsh conditions and produce large amounts of heavy-metal waste. Enzymatic oxidations, on the other hand, operate under relatively mild conditions and produce little if any waste. In the present work, we describe several steps in the oxidative pathway leading from *p*-xylene to terephthalic acid that are catalyzed by enzymes. Chloroperoxidase (CPO) from *Caldariomyces fumago* was used to oxidize *p*-xylene. However, only one of the two aromatic methyl groups was oxidized. To examine the route from 1,4-benzenedimethanol (1,4-BDM) to terephthalic acid, we investigated numerous peroxidase and oxidase enzyme systems. A combination of two enzymes, CPO and xanthine oxidase (XO), was found to produce the highest yield of terephthalic acid from 1,4-BDM. Oxidation of 1,4-BDM to a mixture of predominantly terepthaldicarboxaldehyde, 4-carboxybenzaldehyde, and 4-hydroxymethylbenzaldehyde was carried out by CPO with the continuous addition of hydrogen peroxide as an oxidant. Subsequent addition of XO resulted in a 65% yield of terephthalic acid. A tandem system in which both CPO and XO were present enabled the initial H_2O_2 to be enzymatically regenerated an average of 1.6 times. However, much lower final yields (ca. 2%) of terephthalic acid were obtained.

Keywords: Hydrogen peroxide regeneration; 1,4-Benzenedimethanol; Enzymatic oxidation; Xanthine oxidase; Chloroperoxidase

1. Introduction

The chemical oxidation of hydrocarbons is an important transformation that produces feedstock for the polymers and specialty chemical industries. For example, over 15 million tonnes of terephthalic acid are produced annually from the oxidation of *p*-xylene, a valuable industrial precursor [1]. The current route for commercial production of terepthalic acid from *p*-xylene involves liquid-phase oxidation by a homogenous cobalt and manganese catalyst [1]. The process typically requires temperatures above 190 °C and utilizes titanium-lined reactors. In contrast, biological catalysts are capable of oxidizing many hydrocarbons under relatively mild conditions. Because biological catalysts typically have higher substrate specificities, multiple oxidative steps may necessitate multiple enzymes. Our goal in the present work was to explore pathways for the biocatalytic oxidation of *p*-xylene to terephthalic acid (Scheme 1) utilizing the fewest enzymes.

The search for enzymes capable of catalyzing the complete oxidation of the two aromatic methyl groups

* Corresponding author. Tel.: +1-510-642-2408; fax: +1-510-643-1228.
E-mail address: clark@cchem.berkeley.edu (D.S. Clark).
[1] Present address: School of Chemical Engineering, Purdue University, W. Lafayette, IN 47907-1283, USA.
[2] Present address: Dionex Corporation, Sunnyvale, CA 94085, USA.

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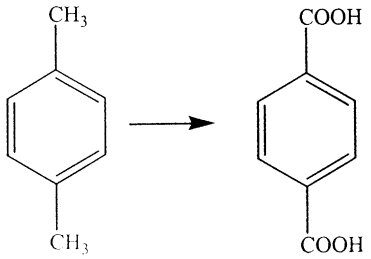

Scheme 1.

of xylene presents several possibilities. One option would be to use a P-450 mono-oxygenase or xylene oxygenase from a micro-organism previously shown to bio-transform *p*-xylene [2,3]. However, utilization of these enzymes in vitro is problematic, as each of these enzymes requires an expensive co-factor and the presence of additional enzymes involved in electron transport. Alternatively, the creation of a microbial pathway for the multiple oxidations required may be possible [4,5]. For example, a bacterial xylene mono-oxygenase enzyme catalyzed the oxidation of toluene to benzaldehyde [6]. However, since the potential application is an industrial, bulk chemical scale process, we desired to avoid the use of micro-organisms or enzymes requiring expensive co-factors.

Peroxidase or oxidase enzymes that use hydrogen peroxide or oxygen as the oxygen source are promising biocatalysts because they do not require costly co-factors. The initial oxidation of *p*-xylene would require an enzyme with mono-oxygenase activity. Based upon the results of similar transformations, such as the oxidation of toluene [7], chloroperoxidase (CPO) from *Caldariomyces fumago* was identified as the most promising enzyme. Of critical importance, CPO has been found to possess P-450 mono-oxygenase like activity [8]. CPO from *C. fumago* is a monomeric heme protein with a molecular weight of approximately 42,000 Da [9]. It has been widely used as a catalyst for a variety of reactions ranging from halogenation [10,11] to epoxidation [12,13], sulfoxidation [14], and oxidation of alcohols to aldehydes [15].

In the present paper, we describe results from the oxidation of *p*-xylene and identification of intermediates in the pathway leading to terephthlalic acid. The end result was a unique enzymatic system for catalysis, in which CPO combined with a microbially-derived XO were applied in tandem to catalyze the oxidation of 1,4-benzenedimethanol (1,4-BDM) to terephthalic acid.

2. Experimental

2.1. Chemicals and enzymes

Chloroperoxidase (EC 1.11.1.10) from *C. fumago*, soybean peroxidase (SPO), bromoperoxidase (BPO) from *Corallina officinalis*, galactose oxidase (GO, E.C. 1.1.3.9) from *Dactylium dendroides*, alcohol oxidase (E.C. 1.1.3.13) from *Pichia pastoris* and *Candida boidinii*, catalase (E.C. 1.11.1.6) from bovine liver and xanthine oxidase (XO, EC 1.1.3.22) from buttermilk and an unidentified micro-organism were purchased from Sigma (St. Louis, MO). The chemicals 1,4-benzenedimethanol, 4-hydroxymethylbenzaldehyde, terephthaldicarboxyaldehyde (TDA), 4-hydroxymethylbenzoic acid, 4-carboxybenzaldehyde, and terephthalic acid were purchased from Aldrich. H_2O_2 test strips were obtained from Ben Meadows Company (Atlanta, GA) and used to determine the concentration of H_2O_2 during the reactions. HPLC grade MeOH and MeCN were purchased from Fisher Scientific.

2.2. Apparatus

A single-syringe infusion pump from KdScientific (Fisher Scientific) was used for the continuous delivery of H_2O_2 (Fisher Scientific). The equipment used for LC analysis included an Agilent Technologies (Wilmington, DE) 1100 liquid chromatograph with photodiode array detector.

2.3. Oxidation of p-xylene

Chloroperoxidase (1650 U) was added to 5 ml of phosphate buffer (pH 6.0) saturated with *p*-xylene (ca. 1.2 mM). The reaction was initiated by the addition of 1.0% aqueous solution of H_2O_2 at 50 μl/h. An additional 1650 U of CPO were added after 8 h. The reaction mixture was magnetically stirred under ambient conditions. After 1 day, the reaction was sampled (0.1 ml) and quenched with 0.9 ml of MeOH, which precipitates the enzyme. After vortexing, the sample

was centrifuged at 18,000 × *g* for 2 min. The supernatant was analyzed by HPLC.

2.4. Enzyme screening

Several peroxidase enzymes were screened for the ability to catalyze the oxidation of 1,4-BDM. The enzymes CPO (1560 U), BPO (100 U) and SPO (51 mg solid) were added to 4 ml of 10 mM 1,4-BDM buffered solution at pH 6.0. The reactions were shaken at 250 rpm and 30 °C. In separate experiments, alcohol oxidases from *P. pastoris* (5 U/ml) and *C. boidinii* (6 U/ml) were screened for the oxidation of 10 mM 1,4-BDM at pH 8.0. The solutions were bubbled with oxygen prior to initiation of the reaction. Catalase (500 U/ml) was included to degrade any H_2O_2 generated. Initial rate data were determined by monitoring the disappearance of substrate over several hours (<10% conversion) with HPLC.

GO and XO from buttermilk and the micro-organism were screened for the oxidation of a 10 mM terephthaldicarboxyaldehyde solution. GO (50 U/ml) was assayed at pH 6.0 while both the forms of XO (5 U/ml) were used at pH 8.0.

2.5. Effect of pH on enzyme activity

Oxidations of 1,4-BDM were performed over a pH range from 3.0 to 8.0. In a typical reaction, CPO (1560 U) was added to 4 ml of a 10 mM 1,4-BDM solution. To initiate the reactions, 3% H_2O_2 was added to a final concentration of 0.33 mM, and an equivalent amount was added every 30 min for 8 h. The oxidation of a 10 mM terephthaldicarboxyaldehyde solution was performed in a volume of 2 ml buffered over a pH range from 5.0 to 9.0. The vials were shaken at 250 rpm and at 30 °C. The rate of substrate disappearance was monitored by UV detection after separation from the products by HPLC.

2.6. Two-step enzymatic oxidation

The oxidation of 1,4-BDM was carried out in a sequential way as follows: CPO enzyme (4650 U) was added to 2 ml of 10 mM 1,4-BDM (pH 4). A 3% aqueous solution of H_2O_2 was added via a micro-syringe at a rate of 50 μl/h. The reaction was performed at 37 °C with magnetic stirring. After 2 days, the pH of the reaction mixture was adjusted to 8.0, followed by addition of 10 mg catalase for removal of any excess H_2O_2 in the system. An amount of 10 U of XO was then added and the solution was purged with pure oxygen. The glass vial was sealed and placed on a shaker at 250 rpm and 30 °C. The reaction mixture was sampled (50 μl) after 1 and 2 days. The sample was mixed with 0.95 ml methanol (HPLC grade), sonicated for 20 min, and centrifuged at 18,000 × *g* for 2 min.

2.7. Chemical oxidations

To assess whether any direct oxidation occurred upon addition of H_2O_2, the following control experiments were performed. Three percent H_2O_2 was added at a rate of 50 μl/h to individual 2 ml potassium phosphate buffer solutions containing 10 mM 1,4-BDM, terephthaldicarboxyaldehyde, and 4-carboxybenzaldehyde. The reactions were performed under magnetic stirring at 37 °C.

2.8. Tandem enzyme system

Two ml of a 10 mM 1,4-BDM solution containing CPO (4650 U) and XO (10 U) was prepared in phosphate buffer, pH 6.0. At the beginning of the reaction, 3% H_2O_2 was added to a final concentration of 1 mM. After capping, the reaction vial was purged with oxygen using a needle inserted through a gas-tight septum. The reaction was incubated at 30 °C and shaken at 250 rpm. In a separate experiment performed at 37 °C, 3% H_2O_2 was added continuously at 50 μl/h with magnetic stirring.

2.9. HPLC analysis

The HPLC method was modified from a previously published method [16]. A Chrompak Chromsep Spherisorb ODS-A column (4.6 × 250 mm) was employed. The aqueous medium was 0.1% (v/v) phosphoric acid buffer, and the injection volume was 20 μl. All solvents used for LC analysis were filtered through a 0.22 μm membrane. A three-component linear gradient program was used to facilitate the separation of the oxidative products (Table 1). The flow rate was 0.8 ml/min. To re-equilibrate the column, the initial solvent composition was maintained for 10 min prior to the next injection. Two wavelengths

Table 1
HPLC gradient program used for the separation and analysis of 1,4-BDM and oxidative products

Time (min)	Aqueous buffer (%)	Acetonitrile (%)	MeOH (%)
0	95	5	–
5	95	5	–
15	55	35	10
20	35	55	10
25	95	5	–

were selected for detection, 220 nm for 1,4-BDM and 254 nm for all oxidized products. Separation and detection were carried out at 30 °C.

3. Results and discussion

3.1. Oxidation of p-xylene

The oxidation of *p*-xylene was investigated using CPO under similar conditions to the previously reported oxidation of toluene [7]. To produce a high final yield of products, a continuous feed of H_2O_2 was employed [17]. The results of the oxidation are as shown in Fig. 1. One methyl group was fully oxidized to the carboxylic acid, producing *p*-toluic acid in 5.2% yield. In addition, 4-methylbenzyl alcohol and *p*-tolualdehyde were also formed in yields of 40.5 and 35.0%, respectively. However, LC/MS analysis of the products provided no evidence for the oxidation of the second methyl group. Thus, as an alternative approach, enzymes were screened for the ability to oxidize 1,4-BDM to terephthalic acid.

Table 2
Enzymes assayed for the oxidation of 1,4-BDM

Enzyme	Rate of 1,4-BDM disappearance (μM per day per mg protein)
Chloroperoxidase	
Caldariomyces fumago[a]	770 ± 250
Bromoperoxidase	
Soybean[a]	14 ± 4.7
Corallina officinalis[a]	960 ± 220
Alcohol oxidase	
Pichia pastoris[a]	200[b]
Candida boidinii[a]	70[b]

[a] This represents the source of enzyme.
[b] Performed in a separate experiment without replication. Pure O_2 was sparged in the liquid medium prior to initiation of reaction.

3.2. Enzyme screening for 1,4-BDM oxidation

Several enzymes, including CPO, SPO, BPO from *C. officinalis*, and alcohol oxidases from *C. boidinii* and *P. pastoris* were examined for the ability to catalyze the oxidation of 1,4-BDM. Table 2 presents a comparison of enzyme activities measured over a 24-h period. The rate of substrate disappearance was highest for CPO and BPO. The alcohol oxidase enzymes, while not requiring external addition of H_2O_2, had substantially lower activity than the peroxidases. Therefore, CPO was selected as the enzyme best suited to oxidize 1,4-BDM, based on its activity and relatively low cost. Furthermore, at the end of each reaction significant amounts of 4-carboxybenzaldehyde and terephthaldicarboxyaldehyde remained (data not shown). Therefore, other enzymes that are well known to oxidize aldehydes were screened to complete the bio-transformation of 1,4-BDM to terephthalic acid.

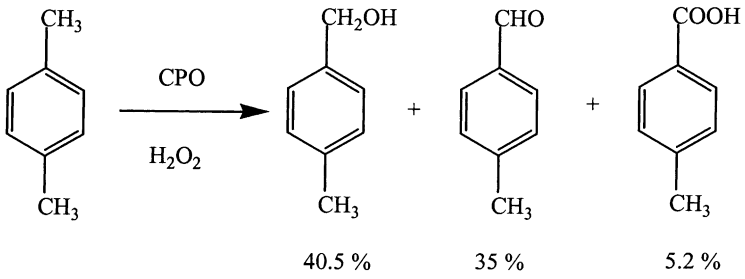

Fig. 1. Oxidation of *p*-xylene by chloroperoxidase (CPO) from *Caldariomyces fumago* at 30 °C and pH 6. The initial concentration of *p*-xylene was 1.2 mM and the feed rate of a 1% H_2O_2 solution was 50 μl/h. After 1 day, the conversion of *p*-xylene was 81%, and the yields of each product (from left, 4-methylbenzyl alcohol, *p*-tolualdehyde, and *p*-toluic acid) were as shown.

Table 3
Enzymes screened for the oxidation of terephthaldicarboxyaldehyde (TDA)

Enzyme	Rate of TDA disappearance (mM per day per mg protein)
Galactose oxidase	
Dactylium dendroides[a]	0.66 ± 0.11
Xanthine oxidase	
Buttermilk[a]	0.50 ± 0.10
Microbial[a]	4.40 ± 0.49

[a] This represent the source of enzyme.

3.3. Enzyme screening for aldehyde oxidation

The results from the oxidation of terephthaldicarboxyaldehyde, a model substrate, by XO from different sources and galactose oxidase are presented in Table 3. The microbial XO had significantly higher activity ($P < 0.001$) than the other enzymes. Therefore, the microbial XO was used for the remaining experiments.

3.4. Effect of pH on enzyme activity

The initial enzymatic activity was examined as a function of pH for CPO and microbial XO. The results are presented in Fig. 2. Each enzyme displayed activity over several pH units, but had vastly different optima. The highest activities for CPO and XO were observed at pH 4.0 and 9.0, respectively. Therefore, pH 6.0 was selected as a pH at which both the enzymes should be simultaneously functional.

3.5. Two-enzyme oxidations

As CPO and XO have different pH optima, we were interested in first demonstrating high yields of terephthalic acid from 1,4-BDM in a two-stage system, with each enzyme operating at its own respective pH optimum. The initial reaction mediated by CPO at pH 4.0 gave rise to 100% conversion of 1,4-BDM after 2 days, where the major products were terepthaldicarboxaldehyde, 4-carboxybenzaldehyde, and 4-hydroxymethylbenzaldehyde (Table 4). After adjusting the pH of the reaction solution to 8.0 and adding catalase to remove excess H_2O_2 remaining in the solution, 10 U of XO were added. Further oxidation of the 1,4-BDM-derived products was subsequently observed, and the final yield of terephthalic acid was 65% (Table 4). Repeating the experiment produced similar yields of all products (data not

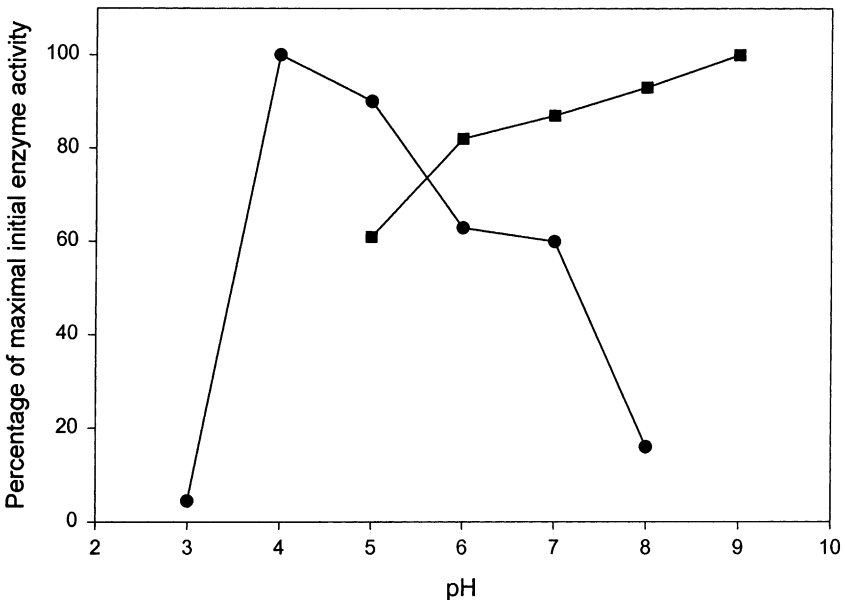

Fig. 2. Initial activity of CPO (circles) and microbial XO (squares) as a function of solution pH. Activity values are plotted as a percentage of each enzyme's maximal initial activity.

Table 4
Oxidation of 1,4-BDM at 37 °C by CPO at pH 4.0 for 2 days, followed by addition of 10 U of XO after adjusting the pH to 8.0

	1,4-BDM	Terephthalic acid	4-Hydroxymethyl-benzaldehyde	4-Carboxybenzaldehyde	Terephthaldicarboxyaldehyde
After CPO (2 days)	0[a]	2	19	58	21
After XO (2 days)	0	65	11	22	0

Ten additional units of XO were added after 72 h. The experiment was terminated after 96 h. H_2O_2 (50 μl/h) was continuously added to the reaction for the first 2 days. The values are given as percentage of sample.
[a] 100% conversion.

shown). After 2 days of the reaction, 30% of the initial CPO activity remained; however, because catalase was present, it is unlikely that the H_2O_2 produced by XO was maximally utilized by CPO.

3.6. Chemical oxidations

To confirm the enzymatic roles of XO and CPO, several controlled reactions were performed. In a XO- and CPO-free system of 10 mM 1,4-BDM to which H_2O_2 was added at a rate of 50 μl/h, a 5% yield of 4-hydroxymethylbenzaldehyde was obtained as the only oxidative product. Two other controlled reactions were performed, i.e. the chemical oxidation of the intermediates terephthaldicarboxyaldehyde and 4-carboxybenzaldehyde. Terephthalic acid was formed in each reaction, 6.0 and 9.5%, respectively, suggesting that the formation of terephthalic acid in the two-enzyme system occurred via both chemical and enzymatic oxidation. However, the chemical oxidation rates were significantly lower (ca. 5–8-fold) than the observed enzymatic rates.

3.7. Tandem enzyme system

In a batch experiment, 1 mM of H_2O_2 was added to a 10 mM 1,4-BDM solution (pH 6) containing both CPO and XO. After 1 day, the yields of 4-hydroxymethylbenzaldehyde, terephthaldicarboxyaldehyde, 4-hydroxymethylbenzoic acid, 4-carboxybenzaldehyde, and terephthalic acid were 7, 0.7, 6, 4 and 2%, respectively. The stoichiometric requirement of H_2O_2 for this product distribution is 2.64 mM. As this value is more than twice the amount supplied, the tandem enzyme system of CPO and XO apparently regenerated some H_2O_2 for the continued oxidation of 1,4-BDM. The yields did not increase after 1 day, demonstrating that the system did not continually recycle every equivalent of H_2O_2 produced. In addition, the final yield of terephthalic acid was unsatisfactory; thus, studies involving the addition of H_2O_2 via continuous feeding were initiated.

To this end, a micro-syringe pump was used to continuously add 3.0% H_2O_2 to the system, following the report of van Deurzen et al. [17]. As shown in Table 5, the resulting yields of oxidation products were enhanced significantly compared to the single H_2O_2-addition experiment.

The main potential advantage of the tandem enzyme system is continuous regeneration of H_2O_2 in the XO-catalyzed reaction. In situ regeneration of H_2O_2 would reduce the amount of exogenous H_2O_2 required and increase the cost-effectiveness of the process. A similar system in which an in situ enzymatic source of H_2O_2 was used was recently reported [18]. In that case, the total turnover number of CPO was increased compared to continuous feeding of H_2O_2.

Table 5
The oxidation of 1,4-BDM by CPO and XO in a tandem system at pH 6.0 and 37 °C

	1,4-BDM	4-Hydroxymethyl-benzoic acid	Terephthalic acid	4-Hydroxymethyl-benzaldehyde	4-Carboxybenzaldehyde	Terephthaldicarboxyaldehyde
1 day	9	13	5	10	31	30
2 days	7	16	6	9	39	25

H_2O_2 (50 μl/h) was continuously added to the system. The values are given as percentage of sample.

In the present study, we have taken the first steps toward the development of an enzymatic system for *p*-xylene oxidation. However, the current system for the biotransformation of *p*-xylene to terephthalic acid is not practical for several reasons. The principle limitation is that the second methyl group of *p*-xylene could not be oxidized. Moreover, CPO is irreversibly inactivated by H_2O_2 [19]. In principle, these limitations may be overcome by employing protein engineering techniques, such as site-directed mutagenesis or directed evolution. Recently, the substrate inhibition of CPO by terminal alkenes was significantly reduced using a directed evolution approach [20]. In addition, site-directed mutagenesis was used to alter the regio-specificity of an aromatic mono-oxygenase [3]. Hence, both these techniques have the potential to alter the substrate specificity of CPO so that the second methyl group of *p*-xylene might be oxidized. Other properties of CPO that could be potentially modified include its pH optimum and catalase-type activity.

Another major disadvantage of the current tandem enzyme system is the limited solubility of *p*-xylene in water. Preferably, the enzymes would be used in a low-water environment with a higher *p*-xylene concentration and a higher volumetric productivity. Recently, CPO has been shown to exhibit high activity in organic solvents, such as isopropanol [21]. The oxidation of benzyl alcohol to benzaldehyde by CPO in *iso*-octane was also recently reported [15]. We performed preliminary experiments to determine the feasibility of using the current enzymatic system in organic solvents. With CPO prepared by salt-activation [22], and dissolving the lyophilized CPO in buffer before adding it to the organic solvent [21], we examined the enzyme's activity in acetone with continuous feeding of 3% H_2O_2. Under these conditions only limited yields of the first oxidation product, 4-hydroxymethylbenzaldehyde, were observed. Moreover, the CPO was rapidly inactivated, and no activity remained after 24 h. By comparison, salt-activation of XO by 99% (w/w) KCl enhanced the oxidation of 10 mM terephthaldicarboxyaldehyde in butyl acetate by 18-fold compared to salt-free enzyme after 1 day. However, even in their activated forms, the activity of both enzymes in organic solvents compatible with the solubility constraints of the substrates and products was too low to warrant further investigation.

Acknowledgements

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