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# Versatile peroxidase degradation of humic substances: Use of isothermal titration calorimetry to assess kinetics, and applications to industrial wastes

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## ABSTRACT

The kinetic constants of a hybrid versatile-peroxidase (VP) which oxidizes complex polymeric humic substances (HS) derived from lignin (humic and fulvic acids) and industrial wastes were determined for the first time using isothermal titration calorimetry (iTC). The reaction conditions were manipulated to enable manganese-peroxidase (MnP) and/or lignin-peroxidase (LiP) activities to be evaluated. The peroxidase reactions exhibited varying degrees of product inhibition or activation; properties which have not previously been reported for VP enzymes. In contrast to previous work (Ertan et al., 2012) on small non-polymeric substrates ($MnSO_4$, veratryl alcohol and dyes), all kinetic plots for polymeric HS were sigmoidal, lacked Michaelis–Menten characteristics, and were indicative of positive cooperativity. Under conditions when both LiP and MnP were active, the kinetic data fitted to a novel biphasic Hill Equation, and the rate of enzymatic reaction was significantly greater than the sum of individual LiP plus MnP activities implying synergistic activation. By employing size-exclusion chromatography and electrospray ionization mass spectrometry, the characteristics of the oxidative degradation products of the HS were also monitored. Our study showed that the allosteric behaviour of the VP enzyme promotes a high level of regulation of activity during the breakdown of model and industrial ligninolytic substrates. The work was extended to examine the kinetics of breakdown of industrial wastes (effluent from a pulp and paper plant, and fouled membrane solids extracted from a ground water treatment membrane) revealing unique, VP-mediated, kinetic responses. This work demonstrates that iTC can be successfully employed to study the kinetic properties of VP enzymes in order to devise reaction conditions optimized for oxidative degradation of HS present in materials used in a wide range of industries.

## 1. Introduction

Humic substances (HS) are formed from microbial degradation of dead plant matter, particularly from lignin. Based on their extraction properties, HS include: humin, insoluble at any pH; humic acid (HA), soluble in alkali and insoluble in acid; and fulvic acid (FA), soluble at all pH values. These components can vary in colour and molecular weight from light yellow and 2 kDa (FA) to black and 300 kDa (humin) (Stevenson, 1994).

Plant lignin is highly resistant to chemical and biological breakdown due to its structure consisting of heterogeneous branched mono-lignol units polymerized via ether and carbon–carbon bonds (Wong, 2009; Niladevi, 2009; Ruiz-Dueñas and Martínez, 2009;

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*Abbreviations:* HS, humic substances; FA, fulvic acid; HA, humic acid; PPE, paper and pulp effluent; FMS, fouled membrane solids; VP, versatile-peroxidase; VP-BA, VP from *Bjerkendera adusta*; LiP, lignin-peroxidase; MnP, manganese-peroxidase; LiP-MnP, combined LiP and MnP activity; iTC, isothermal titration calorimetry; HOD, heat of dilution; SIM, single injection mode; LRET, long range electron transfer; SEC, size-exclusion chromatography.

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Abdel-Hamid et al., 2013). Lignin derived HS tend to be polydisperse polymers of aromatic and aliphatic units that have formed from the polymerization of intermediate lignin degradation products (Wong, 2009; Abdel-Hamid et al., 2013). As a physically and chemically heterogeneous class of structurally complex biopolymers (Gaffney et al., 1996; Niladevi, 2009), HS are often present in soil, marine and ground water environments, and waste water from industrial and municipal water treatment (Gaffney et al., 1996; Duarte et al., 2003; Niladevi, 2009; Abdel-Hamid et al., 2013).

In the pulp and paper industry, HS that are formed from the chemical treatment of wood tend to foul membrane filters during waste water treatment, and cause filter blockage and decrease filtration flux rates (Duarte et al., 2003; Tang and Leckie, 2007; Sutzkover-Gutman et al., 2010). Costs associated with membrane cleaning and the associated loss of productivity from flux rate decline, reduced product outputs and membrane replacement, mean that there is good scope to improve cleaning methods, particularly through the use of enzymes to achieve more environmentally friendly cleaning procedures (Maartens et al., 2002; Cavicchioli et al., 2011).

Versatile peroxidase (VP, EC 1.11.1.16) from white-rot fungi exhibits both lignin peroxidase (LiP, EC 1.11.1.14) and manganese peroxidase (MnP, EC 1.11.1.13) activities (Ruiz-Dueñas et al., 2009; Ertan et al., 2012; Abdel-Hamid et al., 2013; Carabajal et al., 2013). The mechanism and catalytic cycle of LiP, MnP and hybrid VP enzymes involving compounds I, II and III, MnP and LiP long-range electron transfer (LRET) pathway have been determined in detail (Wong, 2009; Ruiz-Dueñas et al., 2009; Bao et al., 2014). Peroxidases catalyze $H_2O_2$-dependent oxidative degradation of aromatic polymers, including HS, by generating radicals which can cleave aromatic rings, ether and C—C bonds, and by causing demethoxylation (Wong, 2009; Abdel-Hamid et al., 2013). Substrate oxidation by peroxidases can also cause radical-radical coupling resulting in spontaneous polymerization (Salvachúa et al., 2013). Due to these properties the biotechnological potential of peroxidases in industries employing lignocellulosic materials has been described (López et al., 2007; Niladevi, 2009; Ruiz-Dueñas and Martínez, 2009; Cavicchioli et al., 2011; Yadav et al., 2011; Wang et al., 2013; Abdel-Hamid et al., 2013).

The oxidation/degradation kinetics of HS has traditionally been determined using spectrophotometry to measure aromatic content (254 and 280 nm) and colour (436 nm), or by measuring total organic carbon. However, values for rate-constants determined this way have been found to vary depending on the method used (e.g. total organic carbon vs $A_{254}$) and the source of HS (Uyguner and Bekbolet, 2005). Measuring HS oxidation by UV–vis spectrophometry can also be impaired by interference from other aromatic compounds, be time-consuming, and lack sensitivity, and fluorescence methods can be affected by pH and ionic strength of assay solutions (Saadi et al., 2006).

In addition to colorimetric methods, a calorimetric method was developed for studying the kinetics of VP from *Bjerkendera adusta* (VP-BA) using simple substrates methylene blue and veratryl alcohol (LiP); $MnSO_4$ and 3-methyl-2-benzothiazolinone hydrazone plus 3-(dimethylamino) benzoic acid (MnP) (Ertan et al., 2012). The study showed that isothermal titration calorimetry (iTC) was useful for determining kinetic properties of both LiP and MnP activities, with conditions developed to enable LiP activity (pH 3.5, absence of $Mn^{2+}$) to be measured separately from MnP activity (pH 4.5, 2 mM $Mn^{2+}$) or LiP and MnP to both be active (pH 4.0, 0.22 mM $Mn^{2+}$). The reason that MnP is fully active and LiP is inactive (<5%) is that both LiP and MnP compete for the same oxidized heme group, and the MnP active-site is very near the oxidized heme group and hence out competes the LiP LRET (long-range electron transfer) pathway. Therefore, higher concentrations of $Mn^{2+}$ progressively inhibit LiP activity (Ertan et al., 2012). This phenomenon only occurs with a hybrid enzyme such as VP-BA and does not occur with separate LiP and MnP enzymes (Mester and Field, 1998).

When simple substrates are used, VP-BA was found to exhibit monophasic Michaelis–Menten kinetics (Ertan et al., 2012). iTC can be performed using a single injection method (SIM) which delivers substrate in a single pulse, and by using SIM and performing a second injection, the effects of product on the kinetic constants ($k_{cat}$ and $K_m$) were also evaluated. The study revealed that VP-BA was allosterically regulated, involving uncompetitive substrate inhibition of LiP activity and homotropic activation of MnP by $Mn^{2+}$ (Ertan et al., 2012).

Although $H_2O_2$ inactivates VP in the absence of substrate or in the presence of excessive peroxide concentrations by forming compound III, the enzyme is not inactivated in the presence of substrate within optimal peroxide concentrations (Wong, 2009; Ertan et al., 2012). The earlier steps in the LiP and MnP reaction pathways (e.g. oxidation of heme iron with $H_2O_2$ and formation and reduction of compound I) are faster than the subsequent steps (Wong, 2009; Ruiz-Dueñas and Martínez, 2009). The calorimetric assays measure the overall heat of the reaction, however, the kinetics of reaction pathways are limited by the slowest reaction step (Murphy et al., 2010). While the faster steps in a reaction pathway produce heat, it is quickly dissipated before heat from the slowest reaction can be measured. In this way, iTC provides a measure of the change in heat associated with the slowest step in the reaction which is the oxidation of substrates and not the activation of heme by $H_2O_2$. The fact that the kinetic constants for numerous substrates were found to be similar for the calorimetric and colorimetric methods indicate that both methods provide data for the same step in the reaction pathway of VP (Ertan et al., 2012).

In the present study we applied the iTC-based approach (Ertan et al., 2012) to extend understanding of the kinetic basis of VP-BA catalysis, to complex polymeric substrates such as HS and industrial wastes. We assessed the degradative ability and the kinetics of oxidation of VP-BA on HS (both HA and FA), waste-water from pulp and paper mill effluent (PPE) and, an extract of solids removed from a reverse osmosis membrane fouled by ground-water (fouled membrane solids, FMS). Reaction conditions were used to enable LiP and/or MnP activities to be measured, and iTC SIM was employed to gain an understanding of industrially relevant conditions where the effects of product accumulation are important. By assessing for the first time, the kinetics of a VP enzyme on complex natural polymeric substrates and the degradation products derived from HA, we identified novel enzymological characteristics (such as monophasic and biphasic sigmoidal kinetics, product inhibition and synergistic activation) that will be useful for applying VP-BA to the degradation of industrial or agricultural lignocellulosic materials (Niladevi, 2009; Wang et al., 2013; Abdel-Hamid et al., 2013).

## 2. Materials and methods

### 2.1. VP-BA and HS

VP-BA was purchased and processed as described previously (Ertan et al., 2012). Purified Suwannee River FA and HA standards (International Humic Substances Society, USA) were completely dissolved in water to a concentration of 8 g $L^{-1}$. PPE was a gift from an Australian pulp and paper mill and was prepared by drying 825 μl under vacuum and redissolving either in water or 0.1 M tartaric acid/NaOH buffer over a range of pH values. FMS were obtained by scraping solids off a reverse osmosis membrane (1305 $cm^2$) that had been fouled by ground water, suspending the solids in 2 ml water and boiling for 5 min. The highly viscous mixture was made up to 3 ml with water and sonicated using a Branson Sonifier 250 at 40% amplitude for 5 min. The mixture was centrifuged at 4752 *g* for