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Chemical kinetics and interactions involved in horseradish peroxidase-mediated oxidative polymerization of phenolic compounds

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ABSTRACT

The primary objective of this research was to evaluate various factors that affect the reaction rate of oxidative coupling (OXC) reaction of phenolic estrogens catalyzed by horseradish peroxidase (HRP). Kinetic parameters were obtained for the conversion of phenol as well as natural and synthetic estrogens estrone (E_1), 17β-estradiol (E_2), estriol (E_3), and 17α-ethinylestradiol (EE_2). Molecular orbital theory and Autodock software were employed to analyze chemical properties and substrate binding characteristics. Reactions were first order with respect to phenolic concentration and reaction rate constants (k_r) were determined for phenol, E_3, E_1, E_2 and EE_2 (in increasing order). Oxidative coupling was controlled by enzyme–substrate interactions, not collision frequency. Docking simulations show that higher binding energy and a shorter binding distance both promote more favorable kinetics. This research is the first to show that the OXC of phenolics is an entropy-driven and enthalpy-retarded process.

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1. Introduction

Researchers have oxidatively polymerized phenolic chemicals with horseradish peroxidase (HRP) to remediate wastewater [1–4]. Oxidative coupling (OXC) is fast and produces insoluble polymers that can be removed in sedimentation or filtration steps. The HRP-OXC catalytic cycle involves: (1) a hydrogen peroxide-induced transfer of two electrons from the iron (III) residue present at the active site of HRP, (2) a one-electron reduction step in which a phenolic substrate donates an electron to the HRP iron $(IV)^+$ residue, (3) a second one-electron reduction step in which a phenolic substrate donates an electron to the HRP iron (IV) residue, and (4) reaction between the two phenoxy radicals, resulting in the formation of dimers. These reaction products may in turn go on to participate in further coupling cycles, yielding higher order oligomer products. HRP-OXC is not as energy intensive as other advanced oxidation processes (i.e. ozonation), and compared to microbial degradation, HRP-OXC is faster and does not present concerns about metabolite toxicity because the byproducts are not soluble. HRP-OXC now stands as a promising and potentially sustainable option for addressing the presence of phenolic chemicals (including some endocrine disruptors) in water.

Researchers have used molecular orbital theory in an attempt to construct quantitative structure-activity relationships (QSAR) that inform HRP-OXC; these results have produced intriguing but at times inconsistent correlations. Several studies showed varying levels of success in generating correlations between the turnover number (k_{cat}) and energy of the highest-occupied molecular orbital (E_{HOMO}) (i.e. between 0.560 and 0.998 [1,5–8]). Correlations between k_{cat} and the energy of the lowest unoccupied molecular orbital (E_{LUMO}) have also produced mixed results for substituted phenols [6–8]. More recent efforts have accounted for enzyme–substrate binding features. Colosi et al. 2006 [1] found that the HRP reactivity is related to the binding distance with respect to histidine-42 residue of the HRP/substrate binding complex. Colosi et al. 2010 [9] went on to engineer HRP proteins in which the active pocket was opened, and they found that HRP reactivity (i.e. k_{cat}) was reasonably correlated ($R^2 = 0.81$) with predicted binding distances. This previous work highlighted the importance of enzyme–substrate binding features. There are, however, other hitherto undetermined factors which influence enzyme–substrate interaction. These include critical thermodynamics parameters (e.g. enthalpy and entropy of activation) and enzyme–substrate binding energy. It is also important to consider the participation of water molecules in OXC reactions occurring in the aqueous phase [10].

The overall objective of the current work is to examine the kinetics and reaction mechanisms associated with HRP-OXC, and particular attention is directed to issues that inform enzyme–substrate interactions. The specific aims are to evaluate (1) reaction kinetics over a range of temperatures, (2) kinetic limitations, (3) enzyme–substrate interactions, and (4) thermodynamic parameters. Five phenolic substrates (phenol, E_1, E_2, E_3, EE_2) were polymerized during laboratory testing.

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2. Materials and methods

2.1. Experimental overview

The phenolic substrates were spiked into synthetic wastewater held in 150 ml beakers and mixed with magnetic stir bars. The reaction kinetics and orders were determined by obtaining the initial reaction rate over a range of phenolic concentrations (i.e. 1 μM–26 μM). The enthalpy of activation (ΔH^*) and entropy of activation (ΔS^*) were determined with data collected at different temperatures (5 °C, 15 °C, 25 °C, 35 °C). The E_{HOMO} was determined with the Gaussian 03 program and the molecular volume for each substrate was determined by dividing their molecular weights by their respective densities. The enzyme–substrate interactions were simulated with AutoDock 4.2 to determine binding energies and binding distances.

2.2. Materials

The following materials were purchased from Sigma–Aldrich (St. Louis, MO): phenol (CAS 108-95-2), steroidal hormones E_1 (CAS 53-16-7), E_2 (CAS 50-28-2), E_3 (CAS 50-27-1), EE_2 (CAS 57-63-6), hydrogen peroxide (50 wt%, CAS 7722-84-1), extracellular horseradish peroxidase (type I, R_Z = 1.3), polyethylene glycol (CAS 25322-68-3), 4-aminoantipyrine (AAP) (CAS 83-07-8), reagent-grade acetonitrile (CAS 75-05-8), and methanol (CAS 67-56-1).

2.3. Enzyme activity assay

A colorimetric assay was used to measure the HRP activity and concentration. The enzyme activity is proportional to the production rate of a constituent that absorbs light at a peak wavelength of 510 nm and with an extinction coefficient (e) of 7100 M^{-1} cm^{-1}. The assay mixture consisted of 10 mM phenol, 2.4 mM AAP, and 0.2 mM H_2O_2. One unit of activity (U) was defined as the number of micromoles of hydrogen peroxide utilized per minute at pH 7.4 and 25 °C [11]. Absorbance was monitored at 510 nm with a UV–vis spectrophotometer (Spectronic 20, Bausch & Lomb) every 5 s for 1 min following a reaction initiation. All assays were performed in triplicate. Relative standard deviations (RSD) of triplicate measurements were always less than 5%.

2.4. Initial reaction rate

The initial reaction rate was determined for each kinetic test [12]. The HRP-OXC reactions were carried out at 25 °C in 100 ml of phosphate buffer (50 mM, pH = 7.0) using 150 ml beakers with various initial concentrations of substrate and a fixed dosage of HRP and H_2O_2. PEG was added to protect HRP from oxidative damage, as suggested by [13]. Methanol stock solutions were made for E_1, E_2, E_3 and EE_2 at 1 mM, and the reaction mixtures were prepared by diluting the stock solution to the desired concentration (between 1 μM and 26 μM). For phenol, the 1 mM stock solution was made in water. The batch reactors were mixed at 300 rpm with a Teflon-coated magnetic bar at neutral pH. Each reactor contained the appropriate mass of substrate, 10 μM H_2O_2, and 30 mg/l PEG, and the reaction was initiated by adding HRP. The initial HRP activity was 0.37 U/ml. The 10 μM H_2O_2 concentration was selected to obtain a molar peroxide-to-substrate ratio of 2.0–5.0, as suggested by previous work [3,14,15]. During the tests, 2-ml aliquots were taken from the batch reactors every 10 s for the first 20 s, and the reaction was stopped by adding 0.1 ml of 10% phosphoric acid. The acidified samples were then filtered through a 0.45-μm syringe filter (Pall Life Science Inc., Ann Arbor, MI). Each experiment was done in triplicate.

2.5. HPLC analysis of phenolic substrates

The phenolic substrate concentrations were measured with an Agilent 1200 series high-performance liquid chromatograph (HPLC) equipped with an Elipse XDB-C18 column (150 mm × 4.6 mm, 5 μm particle size). The concentrations were determined with UV absorbance (wavelength = 197 nm) with external calibration. The mobile phase consisted of 40% reagent-grade acetonitrile (ACN) and 60% deionized water (DI). The flow rate was 1.0 ml/min. The retention times for each substrate were 3.03 min (phenol), 12.31 min (E_1), 7.27 min (E_2), 2.05 min (E_3), and 10.24 min (EE_2).

2.6. Kinetic and thermodynamic determination

The initial reaction rate (v_a) is related to the substrate concentration as shown in the following equation [12]:

$$v_a = \left| \frac{d[A_0]}{dt} \right| = (k[B_0]^m)[A_0]^n = k_r[A_0]^n \quad (1)$$

where A represents the substrate, B is H_2O_2, k_r is a reaction rate constant, and n is the reaction order. k_r and n were determined by plotting $\log(v_a)$ vs. $(\log[A_0])$. Reaction rate data were also used to determine Michaelis–Menten parameters (k_{cat} and K_m) as described in supplemental information (see Appendix A).

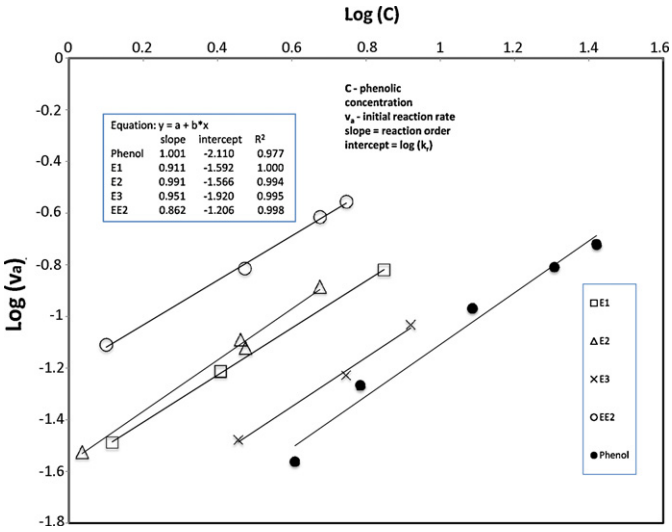

Fig. 1. Initial reaction rates. E_1, E_2, E_3, EE_2, and phenol.

The reaction rate constant was determined at different temperatures using the following [12]:

$$\frac{v_{aT}}{v_{a298\ K}} = \frac{(k_T[B_0]^m)[A_0]^n}{(k_{298\ K}[B_0]^m)[A_0]^n} = \frac{k_{rT}}{k_{r298\ K}} \quad (2)$$

Thermodynamic parameters ΔH^* and ΔS^* were determined using a linear regression of Eyring's equation:

$$\ln \frac{k}{T} = \frac{-\Delta H^*}{R} \times \frac{1}{T} + \ln \frac{k_B}{h} + \frac{\Delta S^*}{R} \quad (3)$$

The Eyring equation was transformed by substituting $k_r = k[B_0]^m$, $[H_2O_2]$ = 10 μM, and m = 1 [16]:

$$\ln \frac{k_r}{T} = \frac{-\Delta H^*}{R} \times \frac{1}{T} + \ln \frac{k_B}{h} + \frac{\Delta S^*}{R} - 9.21 \quad (4)$$

R is the ideal gas constant (8.314 J/mol K), k_B is Boltzmann constant, h is Planck's constant, and T is temperature in Kelvin.

2.7. E_{HOMO} calculation

The E_{HOMO} was determined using the Gaussian 03 program via the Pittsburgh supercomputer center. Structure optimization of the model compound was conducted with 6-31G (d) basis set at level of Unrestricted Hartree–Fock (UHF). After structure optimization, E_{HOMO} of the model compounds were calculated in the same method and basis set.

2.8. Docking simulations

Autodock 4.2 was used to simulate the binding between the five phenolic compounds and HRP. At least ten confirmations were possible for each substrate, and for the purposes of this comparative study, the confirmation that was selected had the lowest binding energy because lower energy states are more stable. The Lammarckian genetic algorithm (GA) method was used to calculate free energy changes. In Autodock 4.2, a docking box of 100 × 100 × 100 points was defined with a grid spacing of 0.375 Å. The structural coordinates of the model horseradish peroxidase compound II (1H55) were downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSBPDB). Then, the crystallographic water molecules were removed from the active site before docking, and the hydrogen atoms and partial charges were added using the Amber force field. Partial charges were assigned to HRP and the phenolic substrates using the Gasteiger Partial Equalization of Orbital Electronegativities method. The coordinates of phenolic substrate were used as the initial position for the docking simulation, and HRP was superimposed onto the phenolic substrate to obtain an initial position. The flexible amino acids residues were HIS42, ARG38, PHE41, and ASN70. The binding distance was between the substrate's phenolic proton and the imidazole δN on the HIS42 residue as suggested previously [1].

3. Results and discussion

3.1. Kinetics

Fig. 1 shows the two-dimensional logarithmic graphs associated with the oxidative coupling reactions carried out in this study. The