Enzyme and Microbial Technology 73–74 (2015) 34–43

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Enzyme catalytic nitration of aromatic compounds

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ARTICLE INFO

Article history:
Received 11 November 2014
Received in revised form 6 March 2015
Accepted 26 March 2015
Available online 11 April 2015

Keywords:
Aromatic compounds
Nitration
Horseradish peroxidase
Enzyme catalysis

ABSTRACT

Nitroaromatic compounds are important intermediates in organic synthesis. The classic method used to synthesize them is chemical nitration, which involves the use of nitric acid diluted in water or acetic acid, both harmful to the environment. With the development of green chemistry, environmental friendly enzyme catalysis is increasingly employed in chemical processes. In this work, we adopted a non-aqueous horseradish peroxidase (HRP)/$NaNO_2$/H_2O_2 reaction system to study the structural characteristics of aromatic compounds potentially nitrated by enzyme catalysis, as well as the relationship between the charges on carbon atoms in benzene ring and the nitro product distribution. Investigation of various reaction parameters showed that mild reaction conditions (ambient temperature and neutral pH), plus appropriate use of H_2O_2 and $NaNO_2$ could prevent inactivation of HRP and polymerization of the substrates. Compared to aqueous–organic co-solvent reaction media, the aqueous–organic two-liquid phase system had great advantages in increasing the dissolved concentration of substrate and alleviating substrate inhibition. Analysis of the aromatic compounds' structural characteristics indicated that substrates containing substituents of —NH_2 or —OH were readily catalyzed. Furthermore, analysis of the relationship between natural bond orbital (NBO) charges on carbon atoms in benzene ring, as calculated by the density functional method, and the nitro product distribution characteristics, demonstrated that the favored nitration sites were the *ortho* and *para* positions of substituents in benzene ring, similar to the selectivity of chemical nitration.

1. Introduction

Nitration, especially aromatic nitration, is a class of very important organic chemical reaction [1], whose products, nitroaromatics, are widely used in explosives [2], medicine [3], pesticide [4] and other industrial fields. At present, nitroaromatics are usually synthesized using a nitration system of concentrated sulfuric acid with nitric acid, producing large amounts of waste water laden with acids and nitroaromatics, which in turn corrode equipment and contaminate the environment. In addition, the described reaction has poor region-selectivity while also producing copious by-products, and is particularly unsuitable for acid-sensitive substrates [5]. Therefore, the traditional manufacturing process needs improvement, where advanced technology is desirable. At present, most of the new processes focus on using of green chemical catalysts for nitration [6,7], and using physical methods such as ultrasonic [8] to increase reaction selectivity.

In recent years, researchers in pathology have found that there are many nitrated products of proteins [9,10] and fatty acids [11,12] in diseased tissues of humans and animals, which nitration is associated with peroxidase in the body. The emergence of this phenomenon also attracted the attention of biochemistry researchers, so began the study of enzymatic nitration. Peroxidase is a kind of heme-containing oxidoreductase which utilizes hydrogen peroxide to oxidize a wide variety of organic and inorganic compounds. Many reactions are catalyzed by peroxidase, such as demethylation [13], epoxidation [14], polymerization [15], coupling [16], and nitration [17]. Horseradish peroxidase (HRP) is one of the most widely used peroxidases, applied primarily as analytical reagent in clinical diagnosis [18] and food chemistry [19]. It has also been widely used as catalyst in biotransformation [20], polymer material synthesis [21] and enzymatic nitration.

Over the years, phenol and phenol derivatives have been the most studied substrates regarding enzymatic nitration by HRP. Dai and co-workers [22] reported the HRP-catalyzed nitration of phenol and *m*-cresol in the presence of hydrogen peroxide and sodium nitrite. In the case of phenol, 4-nitrophenol (14%) and 2-nitrophenol (12%) were the main products obtained. In respect to *m*-cresol, the products obtained were 4-nitro-3-methylphenol (19%) and 2-nitro-5-methylphenol (30%). Similar approaches have

Abbreviations: HRP, horseradish peroxidase; NBO, natural bond orbital.

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http://dx.doi.org/10.1016/j.enzmictec.2015.03.008
0141-0229/

been applied to the nitration of 4-hydroxy-3-methyl acetophenone [17]. The authors reported that the nitro group was directed to the *ortho*-position of the hydroxyl group, being obtained 4-hydroxy-3-methyl-5-nitroacetophenone. Besides the use of simple phenol derivatives, more complex substrates have also been used. For instance, Pezzella and co-workers [23] studied the enzymatic nitration of 17β-estradiol by the HRP/NO_2^-/H_2O_2 system and obtained 2-nitroestradiol, 4-nitroestradiol and 2,4-dinitroestradiol when an excess of nitrite was used. Aromatic amines have also received particular attention. Palumbo and co-workers [24] studied the dopamine oxidation pathways using HRP as catalyst. They found that 6-hydroxydopamine and 6-nitrodopamine were formed by the HRP/H_2O_2/NO_2^- system. Here, the formation of the nitro derivative increased when concentration of NO_2^- increased whereas the formation of 6-hydroxydopamine decreased. On the basis of phenol nitration, Dai and co-workers also reported the synthesis of nitroanilines catalyzed by HRP [25]. 2-Nitroaniline and 4-nitroaniline were found in the nitrated products. 2-Aminotoluene and 4-aminotoluene could also be nitrated to yield corresponding nitrated products.

Although many studies on HRP-catalyzed nitration of aromatic compounds have been reported, current research only focuses on a single class of compounds, such as phenols or amines. Few researchers have investigated the substrate specificity of enzymatic nitration or thoroughly discussed the mechanism of nitro product distribution characteristics. The goals of this work are to determine the group characteristics of aromatic compounds whose nitration can be catalyzed by HRP, and to explore the relationship of charges on carbon atoms in benzene ring, as calculated by the density functional method [26], as well as the feasibility of enzymatic nitration of aromatic compounds and product distribution characteristics. On the basis of experimental results, a database of HRP-catalyzed nitration of aromatics was then established, laying foundation for future research. In consideration of the fact that the majority of relevant aromatic compounds are hydrophobic and purely aqueous reaction systems suffer from poor substrate loadings, a non-aqueous two-phase reaction system was employed. As well, we evaluated the impact of numerous reaction parameters on nitrated product yields, including the concentrations of substrates and H_2O_2, pH, as well as the presence of organic solvents.

2. Materials and methods

2.1. Materials

HRP (300 U/mg) was obtained from MYM Biotechnology Co., Ltd. (Beijing, China). All substituted benzene derivatives and sodium nitrite were obtained from either Sigma–Aldrich or Aladdin. The standard and purity of compounds used for identification of nitration products was GC-grade and all above 97%. Hydrogen peroxide (30% aqueous solution) was purchased from Beijing Chemical Reagent Co., Ltd. (Beijing, China). Methanol and acetonitrile were of HPLC grade and obtained from Fisher Scientific. Milli-Q water was used throughout the study.

2.2. HRP activity and stability assay

The activity of HRP in water–solvent mixtures was determined using spectrophotometer assay [17]. Based on a reaction between 4-aminoantipyrine and phenol in the presence of hydrogen peroxide, this method forms a colored product with absorbance at 510 nm. In brief, a 9.4 mL solution, containing 2.5 mM 4-aminoantipyrine and 170 mM phenol in 20 mM sodium phosphate buffer (pH 7), was added to 2 mL of 8.5 mM hydrogen peroxide. The appropriate volume of the 20 mM buffer was added to give a volume of 19.4 mL. From this volume, 3 mL was drawn and transformed to a colorimetric tube. The reaction was initiated upon addition of 1.5 μL of a 0.1 mg/mL solution of HRP in buffer. HRP activity was measured as the slope of the initial linear portion of the kinetic curve, as determined by recording the increase in absorbance at 510 nm for 5 min at 25 °C. Data was collected using a TU-1810 spectrophotometer, provided from Beijing Purkinje General Instrument Co., Ltd. (Beijing, China).

The stability of HRP under different solvent conditions was assessed by incubating 5 μg/mL HRP for 4 h at 25 °C in 50 mM sodium phosphate buffer (pH 7), containing different concentrations of solvents. The solutions were stirred by ZHWY-2102C Incubator Shaker provided from Shanghai Zhi Cheng Analysis Instrument Manufacturing Co., Ltd. (Shanghai, China). The residual enzyme activity was then measured as described above in the assay system and the results were expressed as a percentage of the activity of enzyme measured in the aqueous assay system in the absence of incubation with the organic solvent.

2.3. Enzymatic nitration

Aqueous–organic co-solvent system (one-phase system). The reaction solution contained 50 mM phosphate sodium buffer (pH 7.0), 5 mM phenol, 100 mM $NaNO_2$ and 10 mM H_2O_2. Methanol was added to the reaction system for a total incubation solution volume of 50 mL, and its concentration was 10% (v/v). The reaction was initiated by addition of enzyme stock solution in the same buffer, to attain a final enzyme concentration of 5 μg/mL. The system was shaken at 25 °C for 4 h.

Aqueous–organic two-liquid phase system (two-phase system). 50 mM phosphate buffer (pH 7.0) was used as aqueous phase which contained 100 mM $NaNO_2$, 10 mM H_2O_2, and 5 μg/mL HRP. The organic phase contained 5 mM substrate. The concentration of organic solvent was maintained at 40% (v/v) and the reaction was initiated by shaking at 25 °C for 4 h.

The influence of different reaction conditions, such as pH, concentrations of sodium nitrite and hydrogen peroxide on HRP-catalyzed nitration in a two-phase system was investigated using phenol as the substrate. Next, aromatic compounds with different substituents (—NH_2, —$NHCH_3$, —$N(CH_3)_2$, —$NHCOCH_3$, —OH, —OCH_3, —CH_3, —F, —Cl, —NO_2, —COOH) were used as substrates to study the substrate specificity of enzymatic nitration.

2.4. Quantitative and qualitative analysis

Sample preparation: The reaction mixtures obtained from above systems were all removed when 4 h after reaction initiated and extracted with acetic ether. The solvent was evaporated under reduced pressure and the 1–2 mL residue was filtered with 0.22 μm microporous membrane filter for analysis by GC–MS, LC–MS and HPLC. The concentrations of the starting material and products were determined from calibration curves constructed using authentic standards.

GC–MS analysis: Qualitative analysis of the above sample was performed on Agilent Technologies 7890A GC system equipped with split/splitless mode and programmed temperature vaporizer inlet, and Agilent 7890A/5975C GC–MS with Triple-Axis Detector, electron impact ionization (EI) (Agilent Corp., MA, USA). The analysis process was carried out on a DB-5 MS chromatographic column (length: 30 m; i.d.: 0.25 mm; coated film thickness: 0.25 μm). The gas chromatographic conditions were: carrier gas was helium (purity >99.999%); the flow rates of carrier gas and backflushing were 1 mL/min and 3 mL/min respectively; the inlet temperature was set at 230 °C; the mode of inlet was split (5:1); the injection volume was 5 μL; the temperature programming was as follows: initial temperature at 60 °C, 10 °C/min to 200 °C, 15 °C/min to 250 °C; the auxiliary heaters was set at 280 °C; total time of analysis was 17.333 minutes. The mass spectrometric conditions: the electron energy and temperature of ion source was set at 70 eV and 280 °C respectively; the monitoring range of m/z was 40–550 m/z; the solvent delay was 3.75 min.

LC–MS and HPLC analysis: An Agilent 6210TOF mass spectrometer with a jet stream electrospray ion source and an Agilent 1200 series fast resolution LC system (Wilmington, DE), Shimadzu LC-15C HPLC (Shimadzu Crop., Kyoto, Japan) with SPD-15C detector were employed for qualitative and quantitative analysis of the nitration products. Agilent Masshunter Workstation A.02.01 was used for system control, data acquisition, and data processing. The monitoring range of m/z was 50–1000 m/z; LC separation was performed on a Kromasil 100-5C18 or Grace Smart RP 18 (250 mm × 4.6 mm) chromatographic column maintained at 55 °C with a flow rate of 0.5 mL/min. The injection volume was 20 μL. Because different substrates or products have different properties, different test conditions were used, including the measuring wavelength, mobile phase and separating column. Table 1 listed the test conditions, for LC–MS analysis, 0.1% formic acid was added to mobile phase of the alkaline sample to ionize the substrate and 0.1% ammonia was added to that of the acid sample, replacing 0.1% trifluoroacetic acid. The mass spectrometer was operated in positive jet stream electrospray ionization (ESI) mode. Nitrogen was used as nebulizer (nebulizer pressure, 25 psi), turbo (heater) gas, curtain, and collision-activated dissociation gas. The capillary voltage was +4000 V and the nozzle voltage was +1000 V. The ion source gas and jet stream gas temperatures were 325 °C, with flows of 10 L/min. The fragmentor was 80 V.

2.5. Computational methods

Full geometry optimization of the 20 substituted benzene derivatives was carried out via density functional theory computations using the Gaussian 03 program package. The theoretical calculations were performed employing the B3LYP functional with the 6-311G (d, p) basis set. The molecular structures of all the compounds were accomplished using GaussView 5.0 program. Natural bond orbital (NBO) charges [27] for the carbon atoms situated in the benzene ring with respect to the substituents were determined.