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Enzymatic nitration of phenols

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Abstract

Soybean peroxidase (SBP), an acidic peroxidase from the seed coat, has been shown to be an effective catalyst for the oxidation of a wide variety of organic compounds including phenols, non-phenolic aromatic compounds, aromatic amines, and polycyclic aromatic hydrocarbons. We recently demonstrated that SBP could also oxidize inorganic compounds, e.g. bromide for the halogenation of aromatic substrates [Enzyme Microb. Technol. 26 (2000) 337]. In the present study, we expand the repertoire of SBP-catalyzed oxidation of inorganic species and demonstrate that SBP can catalyze the nitration of a variety of phenols in the presence of H_2O_2 and sodium nitrite. Using 4′-hydroxy-3′-methylacetophenone as a model phenolic substrate, the influence of various reaction parameters, including the nature of organic co-solvent, pH, and peroxide concentration, on enzyme activity and stability were assessed. Nitration was directed to both *ortho* and *para* positions on the phenol, the latter occurring simultaneously with elimination of the ketone-containing substituent on the ring. Several other phenols were effective nitration substrates including 4-hydroxy-1-indanone, 7-hydroxycoumarin, and 2-hydroxy-5-methylbenzaldehyde. Nitration was also observed with horseradish peroxidase, lipoxidase, lactoperoxidase, chloroperoxidase, and the peroxidase from *Coprinus cinereus*.

Keywords: Phenols; Enzymatic nitration; Soybean peroxidase; Stability; Organic solvents

1. Introduction

Enzymatic methods have become increasingly important in chemical synthesis in the past two decades. Crude and purified enzymes have been used on both laboratory and industrial scale to synthesize value-added compounds which may be difficult to produce using traditional chemical methods. The field of enzymatic synthesis is currently dominated by the use of hydrolases such as lipases, proteases, and glycosidases, mainly due to their ease of use, high stability and activity under synthetic conditions, and their independence from expensive cofactors. The use of oxidative enzymes in synthesis, however, has been more challenging because these enzymes are often less stable and typically require cofactors. Nonetheless, interest in developing processes using oxidative enzymes is increasing. For example, laccases have been used in dye decolorization and in paper and pulp processing [1–3]. Various oxidative enzymes are being used to

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develop methods for bioremediation [4]. Peroxidases have been used in a broad range of reactions including halogenation, hydroxylation, demethylation, sulfoxidation, epoxidation, and polymerization reactions [5–7].

Chemical nitration of phenols is typically performed using dilute nitric acid in water or acetic acid [8]. This can result in a relatively low yield and significant byproduct formation. Conversely, enzymatic nitration is well studied in the context of cell damage by reactive nitrogen species. Lignin peroxidase, when combined with tetranitromethane and hydrogen peroxide has been shown to nitrate veratryl alcohol, 1,4-dimethoxybenzene, and tyrosine [9]. In a rather complex procedure, peroxynitrite, generated from hydrogen peroxide and free nitrite and subsequently purified and stored in base, has been used with myeloperoxidase, superoxide dismutase, and horseradish peroxidase to give nitrated 4-hydroxyphenylacetic acid [10]. Finally, myeloperoxidase, horseradish peroxidase, and lactoperoxidase have been used to oxidize nitrite in the presence of hydrogen peroxide to effect the nitration of both free tyrosine and tyrosine in proteins [11,12].

Herein, we investigate the use of peroxidases in the nitration of phenolic compounds. Selective nitration is important in the preparation of nitroarenes that can be reduced chemically to aminoarenes, which are of use as dyes, as well as in the synthesis of energetic materials. A method for enzymatic nitration similar to that of van der Vliet et al. [11] has been employed, albeit from a synthetic perspective using soybean peroxidase (SBP) to catalyze the nitration of phenols. Specifically, we describe a generally applicable enzymatic system that can be used to nitrate a variety of water- and organic-soluble phenolic substrates, including those that may be acid-labile, and therefore not amenable to chemical nitration. A number of reaction parameters were examined for their effects on the yield of nitrated products, including enzyme, substrate, and H_2O_2 concentration, pH, as well as the presence of organic solvents. Finally, in order to ascertain the general applicability of this approach, we use the optimal conditions for SBP and a model substrate to perform nitration using other peroxidases and on other phenolic substrates.

2. Experimental

2.1. Materials

SBP (82 U/mg), peroxidase from horseradish, type II (170 U/mg), lipoxidase from soybean, type I-S (45,000 U/mg), lactoperoxidase from bovine milk (104 U/mg), and chloroperoxidase from *Caldariomyces fumago* (80 U/mg) were obtained from Sigma (St. Louis, MO). Peroxidase from *Coprinus* sp. was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Substrates and sodium nitrite were obtained from Sigma or Aldrich (Milwaukee, WI). Hydrogen peroxide (30% solution in water) was purchased from Fisher Scientific (Pittsburgh, PA). Solvents were of HPLC grade and obtained from either Aldrich or Fisher Scientific.

2.2. Peroxidase activity assay

The activity of SBP in water-solvent mixtures was determined using a modification of a standard spectrophotometric assay for peroxidase activity [13]. This assay is a reaction between 4-aminoantipyrine and phenol in the presence of hydrogen peroxide to form colored products. In a typical experiment, 0.47 ml of a solution containing 2.5 mM aminoantipyrine and 170 mM phenol in 20 mM sodium phosphate buffer, pH 7, was added to 0.1 ml of 8.5 mM hydrogen peroxide. The appropriate volumes of the buffer and solvent were added to give a volume of 0.97 ml and the desired solvent concentration. The reaction was initiated upon addition of 30 μl of a 6 μg/ml solution of SBP in buffer. Peroxidase activity was measured as the slope of the initial linear portion of the kinetic curve determined by recording the increase in absorbance at 510 nm for 5 min at 25°C. Data were collected using a Shimadzu UV-2101PC spectrophotometer. The results are expressed as a percentage of the activity of the enzyme assayed in aqueous medium.

2.3. Peroxidase stability assay

The stability of SBP under different solvent conditions was assessed by incubating 6 μg/ml SBP for 17 h at 25°C in 20 mM sodium phosphate buffer, pH 7, containing different concentrations of solvents. The residual enzyme activity was then measured as

described above in the assay system that contained the same concentration of the solvent as the incubation medium. The results were expressed as a percentage of the activity of enzyme measured in the aqueous assay system without incubation with the organic solvent. Irreversible inactivation of SBP in different organic solvents was measured by incubating SBP in the presence of co-solvent and the enzyme activity was assayed in the absence of added organic solvent. Again, the results were expressed as a percent of the activity of enzyme measured in the aqueous assay system without incubation with the organic solvent.

2.4. Enzymatic nitration

Optimization of conditions for SBP-catalyzed nitration was carried out using 4′-hydroxy-3′-methylacetophenone as the substrate. The substrate was dissolved in methanol, and $NaNO_2$ and H_2O_2 were then added as solutions in 50 mM sodium phosphate, pH 6–8. The reactions were initiated by the addition of enzyme stock solution in the same buffer, to attain a final enzyme concentration of 100 μg/ml. After 4 h of incubation at room temperature, the sample was removed and extracted with methylene chloride. The solvent was evaporated under reduced pressure and the residue was redissolved in methanol for analysis by HPLC and LC–MS. Reaction mixtures were analyzed on a Shimadzu SCL-10A HPLC system equipped with a Shimadzu SPD-M10A photodiode array detector (detection wavelength 225 nm). A 10 min linear gradient from 100% water to 90% acetonitrile/10% water at a flow rate of 1 ml/min was employed. Trifluoroacetic acid (TFA, 0.1%) was added to all mobile phase components. A Phenomenex Luna C8 (2), 5 μm, 75 mm × 4.6 mm column was used for analyses. The concentrations of the starting material and products were determined from calibration curves constructed using authentic standards, which were either obtained from commercial sources or synthesized as described below.

The reaction conditions identified from the optimization studies were then used for nitration of a range of different substrates. These reactions were analyzed on an LC–MS system including a Perkin-Elmer Series 200 HPLC system equipped with a Perkin-Elmer 785A UV–VIS detector (detection wavelength 225 nm) and a PE-Sciex API 2000 atmospheric pressure chemical ionization mass spectrometer. The column used was a 5 μm Phenomenex Luna C8 (2), 50 mm × 2 mm using a 5 min linear gradient from 5% acetonitrile/95% water to 100% acetonitrile was used, where all solvents contained 0.4% acetic acid.

Enzymatic nitrations were carried out on a preparative scale to characterize the reaction products as described below. All products were purified on a Shimadzu LC8A Preparative Liquid Chromatograph system using a linear gradient from 25% acetonitrile/75% water to 95% acetonitrile/5% water. All solvents contained 0.1% TFA. The column consisted of two serial 7 μm Waters Symmetry Prep C8 radial compression cartridges, 40 mm × 100 mm. Proton NMR spectra were recorded on a Bruker Avance DRX 400. Chemical shifts are reported in ppm (versus tetramethylsilane internal standard).

2.5. Synthesis of 4′-hydroxy-3′-methyl-5′-nitroacetophenone and 2-methyl-4-nitrophenol

4′-Hydroxy-3′-methyl acetophenone (0.5 mmol, 75 mg) was dissolved in a mixture of 5 ml methanol and 34 ml 100 mM phosphate buffer, pH 7. Sodium nitrite (4.86 mmol, 335 mg) was added as a solution in 10 ml buffer, followed by the addition of H_2O_2 (58 μl). The reaction was initiated by adding 5 mg of SBP in 1 ml buffer. After stirring for 4 h at room temperature, the reaction mixture was extracted with 2 × 50 ml methylene chloride and 50 ml ethyl acetate. The organic extracts were combined, dried over sodium sulfate overnight and filtered. The solvent was removed under reduced pressure, and the residue purified by preparative HPLC to give the following two products:

1. 4′-Hydroxy-3′-methyl-5′-nitroacetophenone [14], 48.1 mg (58% yield), $(M - H)^-$ 194, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 11.25 (1H, d, $J = 0.5$ Hz), 8.57 (1H, dd, $J = 2.2$, 0.5 Hz), 8.08 (1H, dt, $J = 2.2$, 0.7 Hz), 2.61 (3H, s) and 2.39 ppm (3H, t, $J = 0.7$ Hz).
2. 2-Methyl-4-nitrophenol [15], 20.7 mg (27% yield), $(M - H)^-$ 152, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.07 (1H, d, $J = 2.7$ Hz), 8.00 (1H, dd, $J = 8.8$, 2.7 Hz), 6.83 (1H, d, $J = 8.$ Hz), 5.68 (1H, s) and 2.32 ppm (3H, s).

2.6. Synthesis of 4-hydroxy-7-nitroindan-1-one and 4-hydroxy-5-nitroindan-1-one

4-Hydroxy-1-indanone (0.4 mmol, 59 mg) was dissolved in a mixture of 8 ml methanol and 4 ml phosphate buffer, pH 7. Sodium nitrite (4 mmol, 276 mg) was added as a solution in 20 ml buffer, followed by the addition of 4 ml of 100 mM hydrogen peroxide in buffer. The reaction was initiated by the addition of 4 mg SBP in 4 ml buffer. After stirring for 4 h at room temperature, the reaction mixture was extracted with 3 × 40 ml ethyl acetate. The combined organic extracts were dried over sodium sulfate and filtered. The solvent was removed under reduced pressure, and the residue purified by preparative HPLC to give the following two products:

1. 4-Hydroxy-7-nitroindan-1-one, 19.4 mg (25% yield), $(M - H)^-$ 192, ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.76 (1H, d, $J = 8.5$ Hz), 7.10 (1H, d, $J = 8.5$ Hz), 2.95 (2H, t, $J = 5.5$ Hz) and 2.73 ppm (2H, m, $J = 5.5$ Hz).
2. 4-Hydroxy-5-nitroindan-1-one, 17.2 mg (22% yield), $(M - H)^-$ 192, ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.90 (1H, d, $J = 8.3$ Hz), 7.20 (1H, d, $J = 8.3$ Hz), 3.05 (2H, t, $J = 5.5$ Hz) and 2.73 ppm (2H, m, $J = 5.5$ Hz).

2.7. Synthesis of 7-hydroxy-8-nitrochromen-2-one and 7-hydroxy-6-nitrochromen-2-one

7-Hydroxycoumarin (0.4 mmol, 65 mg) was dissolved in a mixture of 8 ml methanol and 4 ml phosphate buffer, pH 7. Sodium nitrite (4 mmol, 276 mg) was added as a solution in 20 ml buffer, followed by the addition of 4 ml of 100 mM hydrogen peroxide in buffer. The reaction was initiated by the addition of 4 mg SBP in 4 ml buffer. After stirring for 4 h at room temperature, the reaction mixture was extracted with 3 × 40 ml ethyl acetate. The combined organic extracts were dried over sodium sulfate and filtered. The aqueous phase was concentrated under reduced pressure to dryness, the solids were extracted by sonicating in dry acetonitrile and removed by filtration. All organic extracts from both extractions were then combined and the solvent was removed under reduced pressure, and the residue purified by preparative HPLC to give the following two products:

1. 7-Hydroxy-8-nitrochromen-2-one [16], 34.3 mg (41% yield), $(M - H)^-$ 206, ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.03 (1H, d, $J = 9.6$ Hz), 7.70 (1H, d, $J = 8.8$ Hz), 7.00 (1H, d, $J = 8.8$ Hz) and 6.33 ppm (1H, d, $J = 9.6$ Hz).
2. 7-Hydroxy-6-nitrochromen-2-one [17], 17 mg (20% yield), $(M - H)^-$ 206, ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.43 (1H, s), 8.04 (1H, d, $J = 9.5$ Hz), 6.99 (1H, s) and 6.41 ppm (1H, d, $J = 9.5$ Hz).

2.8. Synthesis of 2-hydroxy-5-methyl-3-nitrobenzaldehyde

2-Hydroxy-5-methylbenzaldehyde (0.4 mmol, 54 mg) was dissolved in a mixture of 8 ml methanol and 4 ml phosphate buffer, pH 7. Sodium nitrite (4 mmol, 276 mg) was added as a solution in 20 ml buffer, followed by the addition of 4 ml of 100 mM hydrogen peroxide in buffer. The reaction was initiated by the addition of 4 mg SBP in 4 ml buffer. After stirring for 4 h at room temperature, the reaction mixture was extracted with 3 × 40 ml of ethyl acetate. The combined organic extracts were dried over sodium sulfate and filtered. The solvent was removed under reduced pressure, and the residue purified by preparative HPLC. 2-Hydroxy-5-methyl-3-nitrobenzaldehyde [18], 18.0 mg (25% yield), $(M - H)^-$ 180, ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.27 (1H, s), 8.11 (1H, dt, $J = 2.4$, 0.7 Hz), 7.88 (1H, dt, $J = 2.4$, 0.7 Hz) and 2.35 ppm (3H, t, $J = 0.7$ Hz).

3. Results and discussion

3.1. Optimization of conditions for SBP-catalyzed nitration

Using 4′-hydroxy-3′-methyl acetophenone as a model substrate, nitration yielded 4′-hydroxy-5′-methyl-3′-nitroacetophenone and 2-methyl-4-nitrophenol as the major reaction products (Fig. 1). In the absence of enzyme, or in the presence of enzyme but in the absence of H_2O_2 or $NaNO_2$, no nitration reaction was observed. Hence, the nitration reaction was clearly enzymatic. Furthermore, C–C bond cleavage occurred simultaneous to nitration. A typical reaction time-course for substrate consumption and product

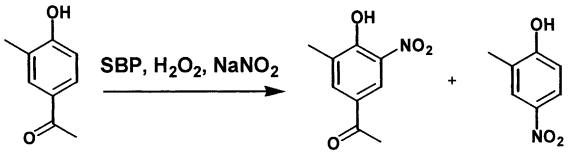


Fig. 1. Nitration of 4′-hydroxy-3′-methyl acetophenone catalyzed by SBP.

accumulation in the nitration of 4′-hydroxy-3′-methyl acetophenone in the presence of 10% methanol (added to improve substrate solubility) is shown in Fig. 2. The reaction reached completion within 8 min, and no significant changes occurred thereafter up to 6 h (data not shown). The termination of the reaction was not due to enzyme inactivation as addition of fresh enzyme at that point did not result in further conversion. Although in most cases, the reaction was completed within 10–30 min, in all subsequent experiments we used the reaction time of 4 h, which was convenient from the standpoint of experimental setup. To optimize this reaction, we examined the influence of several reaction parameters on SBP-catalyzed nitration. These parameters included the presence of organic solvents, pH, and concentrations of H_2O_2 and $NaNO_2$.

3.1.1. Effect of organic solvents on SBP activity and stability

Many potential phenolic substrates for SBP-catalyzed nitration are poorly soluble in water, and

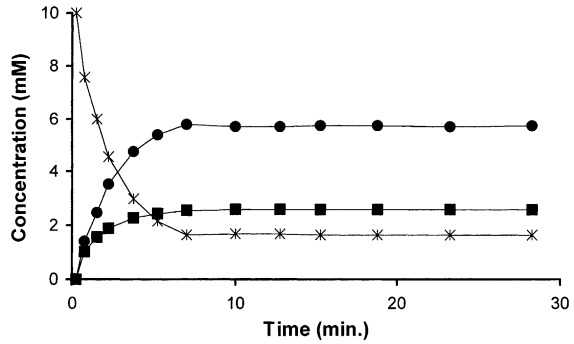


Fig. 2. Time course of SBP-catalyzed nitration of 4′-hydroxy-3′-methyl acetophenone. Reaction conditions: 10 mM 4′-hydroxy-3′-methyl acetophenone, 10% methanol, 10 mM hydrogen peroxide, 100 mM sodium nitrite, 0.1 mg/ml SBP, pH 7. Symbols: (✳), substrate; (●), product of *o*-nitration; (■), product of *p*-nitration.

therefore the presence of organic solvents in the reaction medium is required to achieve the desired level of substrate concentration. The influence of several commonly used organic solvents on the activity of SBP is shown in Fig. 3. A simple peroxidase assay was employed; namely, the oxidation of phenol as measured by the reaction between the phenol oxidation product and aminoantipyrine, which is easily followed spectrophotometrically, and this was deemed to be representative of peroxidase activity, including nitration, in different media. In most cases, the addition of 10% solvent did not significantly affect SBP activity.

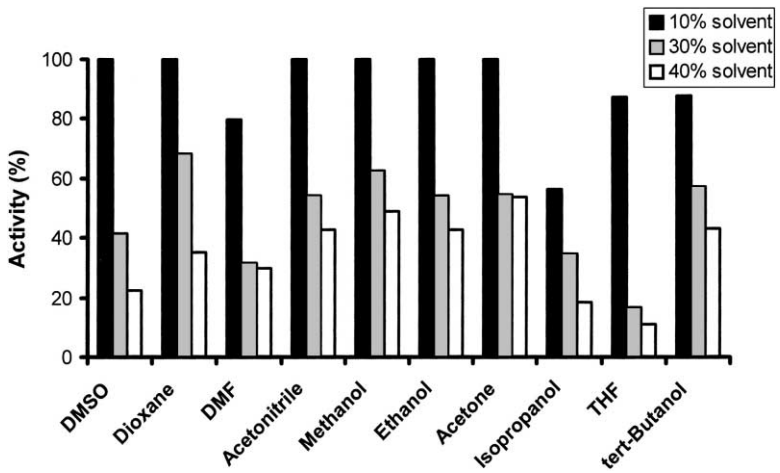


Fig. 3. Effect of organic solvents on the activity of SBP in the reaction between 4-aminoantipyrine and phenol. The initial rate in the absence of added solvents is taken as 100%. Solvents are arranged in order of increasing log *P*.

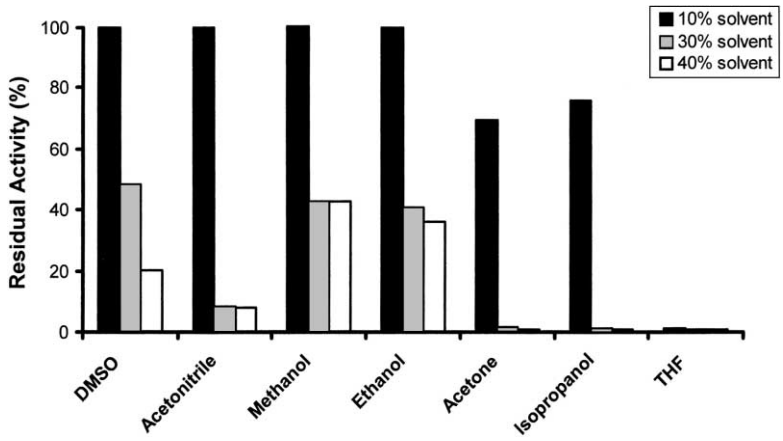


Fig. 4. Residual activity of SBP in the reaction between 4-aminoantipyrine and phenol after incubation in the presence of organic solvents for 17 h at 25°C. The activity was measured in the presence of the same concentration of solvent that was used in the incubation. Solvents are arranged in the order of increasing log *P*.

However, the activity sharply decreased when the solvent concentration was raised to 40%. No obvious correlation was obtained between enzyme activity and solvent parameters, such as log *P*, dielectric constant, dipole moment, or the solubility parameter [19].

SBP was found to be quite stable in aqueous buffer, retaining full activity after 17 h. To assess the stability of SBP against inactivation by organic solvents under synthetically-relevant conditions, the enzyme was incubated in the presence of selected solvents and the residual activity was determined under the same solvent conditions that were used for incubation. The results of these measurements are presented in Fig. 4. The data show that the stability of SBP is strongly dependent on the nature and concentration of the organic solvent. Little or no loss of activity was detected in the presence of 10% methanol, ethanol, dimethylsulfoxide (DMSO), or acetonitrile. In the presence of acetone, the enzyme was less stable, while tetrahydrofuran (THF) caused complete loss of activity. Higher organic solvent concentrations resulted in dramatically lower enzyme stability for acetonitrile, acetone, and THF, while the stability in DMSO, methanol, and ethanol remained high even up to 40% (v/v) organic co-solvent.

The irreversible inactivation of SBP due to incubation in various organic co-solvents was measured by removing the enzyme from the aqueous/organic mixture and placing it in a fresh aqueous buffer solution. As shown in Fig. 5, all solvents caused some irreversible loss in SBP activity. Moreover, and as is consistent with the stability under synthetically-relevant conditions (Fig. 4), the most significant losses in enzyme stability occurred in acetone, isopropanol, and THF, indicating that inactivation in these solvents is mainly irreversible in nature. Conversely, in methanol, ethanol, DMSO and acetonitrile, inactivation is at least partially reversible. As with enzyme activity, there was no obvious correlation between enzyme stability and the physicochemical properties of the solvent used.

3.1.2. Effect of organic solvents on SBP-catalyzed nitration

The presence of different organic solvents in concentrations of up to 30% did not significantly affect the outcome of the nitration reaction in terms of either overall conversion or distribution of products (Fig. 6), despite the fact that some of these solvents (e.g. acetonitrile and acetone) caused enzyme inactivation at higher concentrations (Figs. 3 and 4). Because the nitration reaction reaches completion within a matter of minutes (Fig. 2), the long-term stability of SBP in these solvent mixtures likely has little influence on the nitration yield. In all subsequent experiments, 10% methanol was included in the reaction medium to assist in the dissolution of the substrate.

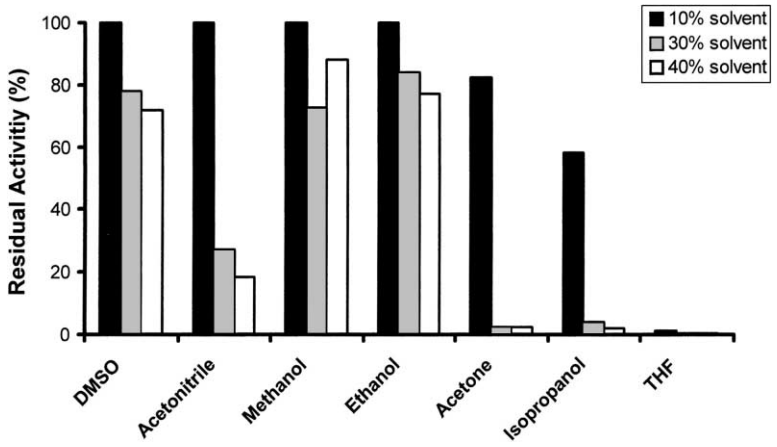


Fig. 5. Residual activity of SBP in the reaction between 4-aminoantipyrine and phenol after incubation in the presence of organic solvents for 17 h at 25°C. The activity was measured in the absence of organic solvent. Solvents are arranged in the order of increasing log *P*.

3.1.3. *Effect of pH*

The highest conversion of the substrate to nitrated products was observed at pH 7.0 (Fig. 7). This result is consistent with the literature, in which pH optima between 2.0 and 7.5 have been reported, depending on the reaction [20–22]. We did not test pH < 6 because, under acidic conditions in the presence of $NaNO_2$ and H_2O_2, non-enzymatic nitration or nitrosation are known to occur [8]. No spontaneous reaction was detected in our experiments within the tested pH range. Above pH 7, the substrate was found to be significantly depleted, but the conversion to the desired nitrated products decreased with increasing pH. This could be explained by the occurrence of a side reaction under alkaline conditions, for example, phenol oxidation to the related dimeric phenol.

3.1.4. *Effect of reactant concentrations*

Nitration of 4′-hydroxy-3′-methyl acetophenone first increased with increasing hydrogen peroxide concentration and then reached a plateau at the point where concentrations of H_2O_2 and substrate were

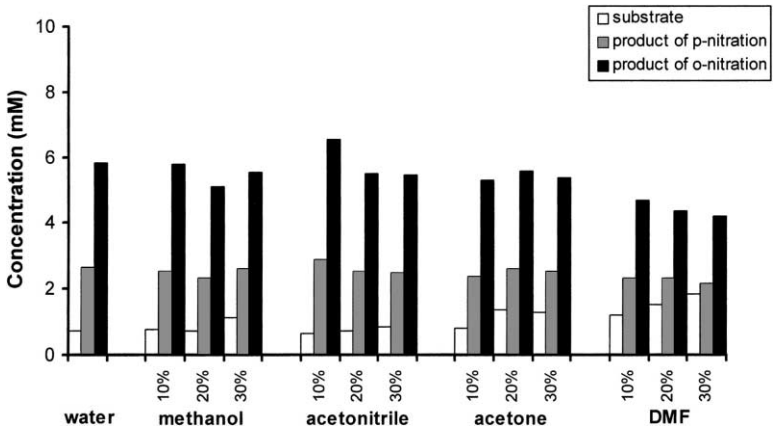


Fig. 6. Effect of organic solvents on substrate conversion and product distribution in SBP-catalyzed nitration of 4′-hydroxy-3′-methyl acetophenone. Reaction conditions: 10 mM 4′-hydroxy-3′-methyl acetophenone, 10 mM hydrogen peroxide, 100 mM sodium nitrite, 0.1 mg/ml SBP, pH 7.

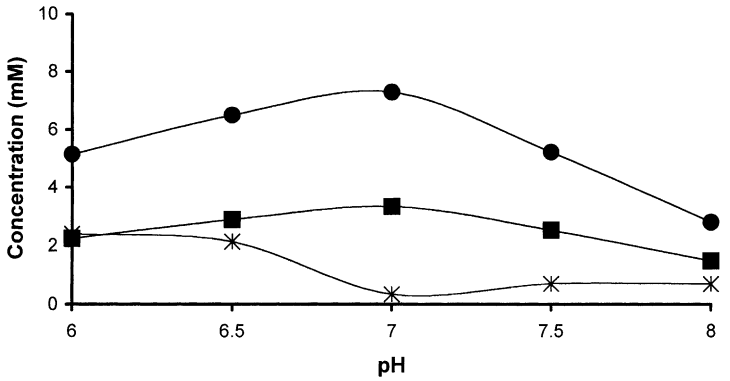


Fig. 7. Effect of pH on SBP-catalyzed nitration of 4′-hydroxy-3′-methyl acetophenone. Reaction conditions: 10 mM 4′-hydroxy-3′-methyl acetophenone, 10% methanol, 10 mM hydrogen peroxide, 100 mM sodium nitrite, 10 mM hydrogen peroxide, 0.1 mg/ml SBP. Symbols: (⁎), substrate; (●), product of *o*-nitration; (■), product of *p*-nitration.

approximately equal (Fig. 8). This indicates that the formation of nitration products and consumption of H_2O_2 occur in a 1:1 stoichiometric ratio. The ratio of 4′-hydroxy-5′-methyl-3′-nitroacetophenone to 2-methyl-4-nitrophenol was 2:1. In all subsequent experiments, equimolar concentrations of the substrate and H_2O_2 were used.

Enzymatic nitration competed with the typical peroxidase-catalyzed polymerization reaction of phenolic substrates [23]. In the absence of $NaNO_2$, oxidative polymerization of the substrate occurred. No polymerization was observed at 10-fold or higher excess of $NaNO_2$. Therefore, in all our experiments, nitrite was used in at least 10-fold molar excess over substrate. A higher excess of $NaNO_2$ did not influence the observed reaction (data not shown).

At 10 mM substrate, any concentration of SBP above 20 μg/ml resulted in reaction rates high enough to complete the nitration within the chosen reaction time (4 h). To ensure completion of the reaction, an enzyme concentration of 100 μg/ml was used in all further experiments.

3.2. Enzymatic nitration of other phenolic compounds

The optimal conditions for SBP-catalyzed nitration identified above were used in the synthesis of nitrated derivatives of a range of phenolic compounds

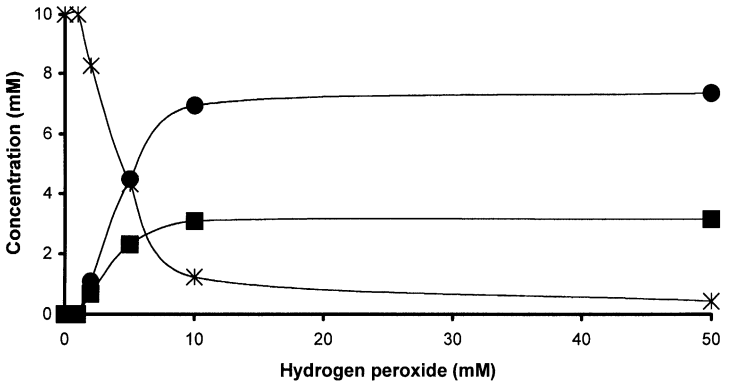
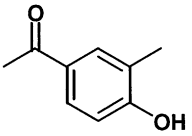
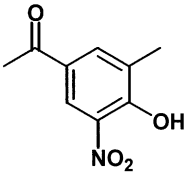
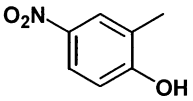
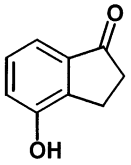
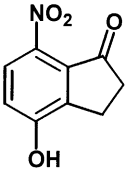
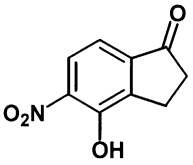
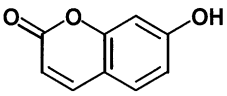
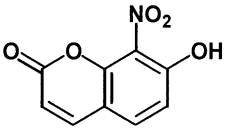
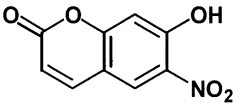
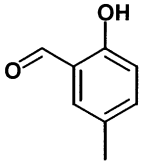
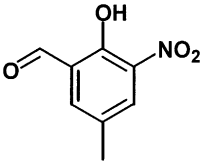


Fig. 8. Effect of hydrogen peroxide concentration on SBP-catalyzed nitration of 4′-hydroxy-3′-methyl acetophenone. Reaction conditions: 10 mM 4′-hydroxy-3′-methyl acetophenone, 10% methanol, 100 mM sodium nitrite, 0.1 mg/ml SBP, pH 7. Symbols: (⁎), substrate; (●), product of *o*-nitration; (■), product of *p*-nitration.

Table 1
Products and isolated yields for substrates which undergo nitration using soybean peroxidase[a]

Starting material	Product 1	Product 2
		
4'-hydroxy-3'-methylacetophenone	58%	27%
		
4-hydroxy-1-indanone	25%	22%
		
7-hydroxycoumarin	41%	20%
		–
2-hydroxy-5-methyl-benzaldehyde	25%	

[a] Reaction conditions were 10 mM substrate, 10 mM hydrogen peroxide, 0.1 mg/ml SBP and 100 mM sodium nitrite in 50 mM sodium phosphate buffer, pH 7, containing 10% methanol.

(Table 1). The identities of the products were confirmed by NMR and LC–MS analyses, as described in Section 2. It was found that the *ortho* and *para* positions of the phenolic ring were the favored nitration sites, similar to the selectivity of chemical nitration [8]. In the absence of $NaNO_2$, all of the phenols shown in Table 1 underwent the standard SBP-catalyzed oxidative coupling and gave unidentified oxidative coupling products. Despite the excess of nitrite, several substrates, including 4-hydroxy-1-indanone, catechin, 1-naphthol, 8-hydroxyquinoline and arbutin, reacted to form an insoluble precipitate, indicating polymer formation. Nitration of 4-hydroxy-1-indanone occurred with roughly equal *ortho* and *para* attack. 2-Hydroxy-5-methyl-benzaldehyde was nitrated solely in the *ortho* position, indicating that the *para* methyl substituent could not be displaced.

3.3. Enzymatic nitration catalyzed by other peroxidases

Several other peroxidases were tested as nitration catalysts under optimal conditions identified for nitration catalyzed by SBP. The data summarized in Fig. 9 show that lipoxidase and peroxidases from horseradish and *Coprinus* sp. were also capable of catalyzing the nitration reaction. Lactoperoxidase was less effective than the other peroxidases, and chloroperoxidase was found to be a poor nitration catalyst under these conditions. This is not surprising as the mechanism of

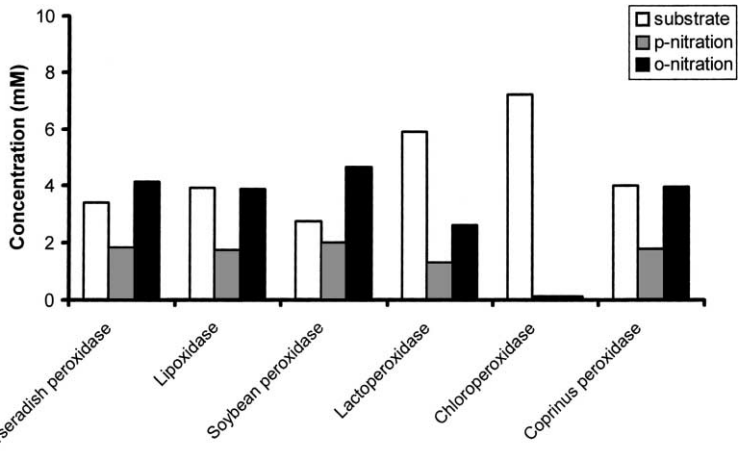


Fig. 9. Nitration of 4′-hydroxy-3′-methyl acetophenone catalyzed by different peroxidases. Reaction conditions: 10 mM 4′-hydroxy-3′-methyl acetophenone, 10% methanol, 250 mM sodium nitrite, 0.1 mg/ml SBP, 10 mM hydrogen peroxide, pH 7.

chloroperoxidase differs from that of the imidazole ligand-containing peroxidases, such as SBP, HRP, and lactoperoxidase [5].

In conclusion, we have shown that SBP is an effective catalyst for the nitration of phenols in the presence of various organic co-solvents at concentrations up to 40% (v/v). The mild reaction conditions (ambient temperatures and neutral pH) make this approach most appropriate for complex compounds (both natural and synthetic) with limited aqueous solubility and with labile functional groups.

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