Guaiacol Oxidation Products in the Enzyme-Activity Assay Reaction by Horseradish Peroxidase Catalysis

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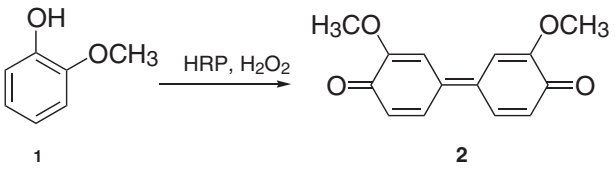
In the horseradish peroxidase (HRP)-catalyzed oxidation of guaiacol (**1**), trimeric products, and diastereomers of biphenoquinones have been newly identified. The oxidation product was analyzed by HPLC, liquid chromatography electrospray ionization mass spectrometry (LC-ESIMS), and ^{1}H NMR spectroscopy.

HRP catalytically achieves one-electron oxidation of a wide variety of aromatic substrates to free radicals, typically phenols to phenoxy radicals, at the expense of hydrogen peroxide.[1,2] Extensive works on the structure and function have been reported, in which oxidation of guaiacol is often utilized for the assay of the enzyme activity.[3,4] As the oxidation product of the reaction, a cyclic tetraguaiacol had been considered, while the visible spectrum can not be accounted for.[5] However, recent studies revealed that 3,3′-dimethoxy-4,4′-biphenoquinone (**2**) is the main product showing absorption maximum around 470 nm (Scheme 1).[6,7]



Scheme 1. Reaction of guaiacol oxidation utilized for the assay of enzyme activity.

HRP catalyzes the oxidative polymerization of a wide variety of phenol derivatives[8,9] and also the degradation of rubbers recently reported.[10] For example, the polymerization of phenol produced soluble polymers with a structure consisting of phenylene and oxyphenylene units.[11] In a series of our investigation on the oxidative polymerization, we report here the detailed study on the oxidation products of guaiacol formed by this important assay reaction, where the production of trimeric products and diastereomers of biphenoquinones was newly found.

When guaiacol was subjected to oxidation with HRP catalysis, the color of the reaction mixture turned red and precipitation occurred.[12] The oxidation products were obtained in 88% yield. The color was sensitive to light and reducing agent. Figure 1 shows HPLC traces of reverse phase separation of the oxidation products. The trace with RI detection (a) shows four main products as peaks A–D. The area ratio of each peak is 16, 44, 19, 13%, respectively. The traces detected by UV absorption at 254 nm (b) and at 470 nm (c) suggest quinone structures for products A and C, and non-quinone structures for B and D. LC-ESIMS of product A revealed a protonated molecule at m/z 245, confirming the structure **2** in accord with the previous result (Scheme 2). Product C exhibiting absorption at 470 nm shows a protonated molecule at m/z 367. This value is consistent with trimeric 3-(4-hydroxy-3-methoxyphenyl)-5,3′-dimethoxy-4,4′-biphenoquinone (**3**). Products B and D were estimated to be 3,3′-dimethoxy-4,4′-biphenol (**4**) and 3-(4-hydroxy-3-methoxyphenyl)-5,3′-dimethoxy-4,4′-biphenol (**5**), respectively, from the molecular ions. These biphenols would be formed via C–C coupling of guaiacol. Subsequent oxidation of the biphenols would produce corresponding biphenoquinones showing absorption at 470 nm. While formation of C–O coupling products and oligomers is possible, the total amount of the other products was only ca. 8% according to the HPLC analysis with RI detection.

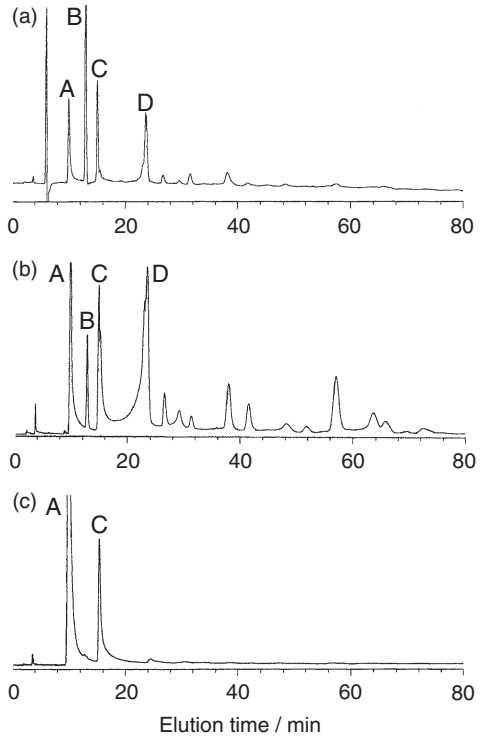


Figure 1. Reverse phase separation of the guaiacol oxidation products. The separation was performed on an ODS-80Ts column using acetonitrile/water (1:1, v/v) eluent at a flow rate of 1.0 mL min^{-1} with (a) RI detection, (b) UV detection at 254 nm, and (c) UV detection at 470 nm.

For further characterization, the reaction mixture was purified by preparative HPLC on an ODS-80Ts column using acetonitrile/water (1:1, v/v) as eluent. Products B and D were readily assigned by ^{1}H NMR to **4** and **5**, respectively.[13,14] On the other

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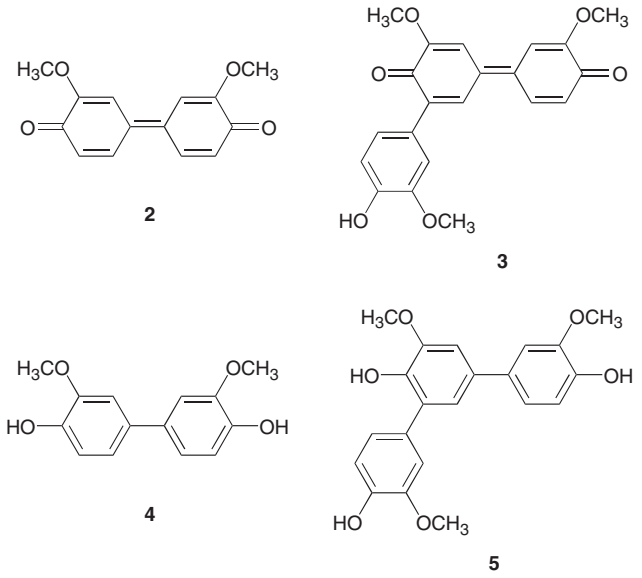
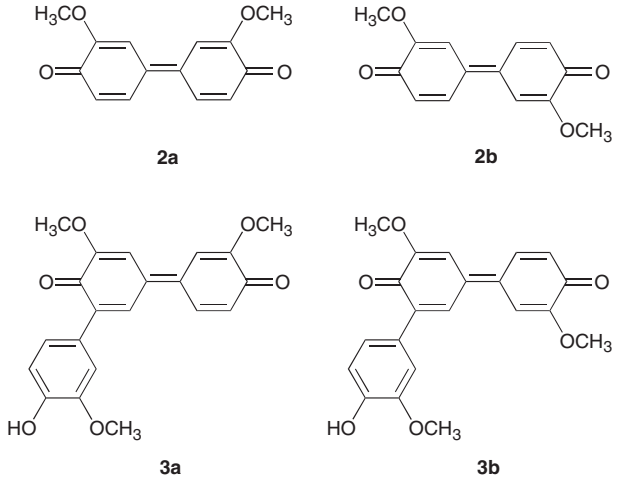


Scheme 2. Structures of guaiacol oxidation products.

hand, ^{1}H NMR spectra indicated that products A and C did not consist of a single product. In the case of product A, multiple signals were observed in the region of δ 6.6–8.0 representing the protons of biphenoquinonic moiety, while two singlet peaks around δ 4.0 were assigned to methoxy protons. Correlation of protons was confirmed by 2D COSY experiments. Considering the structure of **2**, two diastereomers, 3,3′-dimethoxy-4,4′-biphenoquinone (**2a**) and 3,5′-dimethoxy-4,4′-biphenoquinone (**2b**), should be formed as depicted in Scheme 3. In the case of product C, similar signals were observed. Multiple biphenoquinonic protons and methoxy protons were assigned to corresponding trimeric diastereomers, 5-(4-hydroxy-3-methoxyphenyl)-3,3′-dimethoxy-4,4′-biphenoquinone (**3a**) and 3-(4-hydroxy-3-methoxyphenyl)-5,3′-dimethoxy-4,4′-biphenoquinone (**3b**). Formation of trimeric products showing visible absorbance was not negligible even in the enzyme assay conditions, meaning that the formation ratio of the products affects the result of the assay and should be considered for the accurate enzyme assay.

In the HRP-catalyzed oxidation of *o*-cresol, oligomeric precipitates were preferentially obtained instead of biphenoquinones. Characteristic color formation in the oxidation of guaiacol was not attributed to substitution of *ortho* position but to electron-donating methoxy group. One-electron oxidation of guaiacol generates monomeric phenoxy radical, followed by the formation of biphenol via radical coupling and tautomerization. Subsequent oxidation of the biphenol produces phenoxy radical again. Further oxidation of the dimeric phenoxy radical should be preferred in the guaiacol oxidation owing to the electron-donating methoxy group and results in two-electron-oxidized biphenol, leading to the formation of biphenoquinone. On the other hand, radical coupling should be preferred in *o*-cresol oxidation because of lower electron-donating ability of methyl group than that of methoxy group. Thus the electron-donating methoxy substituent and free para position lead to the characteristic feature of guaiacol oxidation.

In summary, the present paper reports formation of trimeric products and diastereomers of biphenoquinones in addition to the hitherto known dimeric products, in the guaiacol oxidation catalyzed by HRP. These results suggest that the currently used HRP-activity assay method is to be reconsidered for the improved accuracy.



Scheme 3. Structures of biphenoquinonic diastereomers.

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References and Notes

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12 Experimental procedures: Guaiacol (5 mmol) and HRP (1 mg) in 25 mL of 0.1 M phosphate buffer (pH 7.0) were placed in a 50-mL flask. Hydrogen peroxide (5% aq. solution, 3.4 mL, 5 mmol) was added dropwise to the mixture for 2 h at room temperature under air. After 3 h, the precipitates were collected by centrifugation and washed with water repeatedly, followed by drying in vacuo to give 0.54 g of the product (yield 88%).
13 Compound **4**: ^{1}H NMR ($CDCl_3$, δ) 3.9 (s, 6 H), 5.6 (s, 2 H), 6.97 (d, 2 H), 7.01 (d, 2 H), 7.03 (dd, 2 H).
14 Compound **5**: ^{1}H NMR ($CDCl_3$, δ) 3.91 (s, 3 H), 3.94 (s, 3 H), 3.99 (s, 3 H) 5.60 (s, 1 H) 5.64 (s, 1 H) 5.81 (s, 1 H) 6.97 (d, 1 H), 6.98 (d, 1 H), 6.99 (d, 1 H), 7.04 (d, 1 H), 7.06 (dd, 1 H), 7.10 (d, 1 H), 7.14 (dd, 1 H), 7.18 (d, 1 H).

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