Short Communication

Structures of the Laccase-catalyzed Oxidation Products of Hydroxybenzoic Acids in the Presence of ABTS [2,2'-Azino-di-(3-ethylbenzothiazoline-6-sulfonic Acid)]

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Recently, enzymatic colorimetric analyses have been widely studied^{1~4)} because the highly specific activities of enzymes are advantageous for the analysis of contaminated samples such as biological fluids and foods.

We reported a new enzymatic colorimetric reaction of phenol derivatives^{5,6)} which could be applied to the quantitative analysis of the activities of angiotensin I converting enzyme,⁷⁾ carboxypeptidase A,⁸⁾ α -glucosidase and α -

amylase.⁶⁾ The assays are based on the oxidative coupling of a substrate, a phenol derivative, with a coupling compound, 2,2'-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 4-aminoantipyrine or 2,6-dibromo-4-aminophenol, in the presence of laccase [benzenediol: oxygen oxidoreductase, EC 1.10.3.2]. No detailed study has been reported on such a laccase reaction with phenol derivatives and a coupling compound, although laccase is known to oxidize several phenolic compounds.^{9~15)} These facts prompted us to examine the mechanism of this new type of colorimetric reaction.

As shown in the previous paper,⁵⁾ ABTS combined with phenol derivatives, hydroxybenzoic acids, in the presence of laccase to form colored compounds which showed stronger absorption than that of the products from 4-aminoantipyrine in the visible range. This paper describes the structural elucidation of the two enzymatic oxidation products obtained on reaction of the coupling compound, ABTS, and the substrates, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid.

The enzyme reactions were carried out at 37°C in 0.1 M sodium phosphate buffer, pH 6.0, containing laccase from *Polyporus versicolor* IFO 9791, ABTS and 3-hydroxybenzoic acid or 4-hydroxybenzoic acid. The two reaction mixtures developed a pink color with an absorption maximum at 515 nm (3-hy-

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droxybenzoic acid) and a strong blue color with an absorption maximum at 590 nm (4-hydroxybenzoic acid), respectively. The reaction mixtures were passed through Toyopearl HW-40 and Toyopearl HW-55 columns to yield colored products I (50%) and II (78%), which were formed from 3-hydroxybenzoic acid and 4-hydroxybenzoic acid, respectively.

Compound I, deep reddish brown powder, mp $> 300^{\circ}\text{C}$, UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (log ϵ): 194 (4.46), 210 (4.44), 295 (3.95), 514 (4.58), was assigned the molecular formula of $\text{C}_{16}\text{H}_{11}\text{N}_3\text{O}_6\text{S}_2\text{Na}_2$ on the basis of the SIMS (MH^+ : m/z 452) and the following evidence. The IR spectrum ($\nu_{\text{max}}^{\text{KBr}}$ cm^{-1}) exhibited bands corresponding to a carboxylate anion (1540 and 1380) and unsaturated ketone (1615) besides benzenoid absorptions (1615 and 1580). The ^1H -NMR spectrum ($\text{DMSO}-d_6$, δ ppm)¹⁶⁾ showed the presence of three aromatic protons and 3-ethyl protons of a 2,3,6-substituted benzothiazoline moiety (7.54, 1H, d, $J=8.5$ Hz, 4'-H; 7.67, 1H, dd, $J=8.5$, 1.4 Hz, 5'-H; 7.96, 1H, d, $J=1.4$ Hz, 7'-H; 1.34, 3H, t, $J=7.2$ Hz, CH_3 ; 4.41, 2H, q, $J=7.2$ Hz, $\text{CH}_2\text{-N}$) and three protons of a 2-carboxy-1,4-benzoquinone moiety (6.00, 1H, d, $J=2.0$ Hz, 2-H; 6.32, 1H, dd, $J=10.1$, 2.0 Hz, 4-H; 8.00, 1H, d, $J=10.1$, 5-H). The presence of 2,3,6-substituted benzothiazoline and 2-carboxy-1,4-benzoquinone moieties was also supported by the ^{13}C -NMR spectral data [$\text{DMSO}-d_6$, δ ppm: 12.54 (CH_3), 110.99 (C-4'), 120.27 (C-7'), 121.64 (C-2), 124.05 (C-7'a), 124.72 (C-5'), 125.33 (C-5), 128.89 (C-4), 139.25 (C-3'a), 143.85 (C-6'), 146.86 (C-1), 152.32 (C-2'), 168.99 (COO^-), 171.80 (C-6), 187.66 (C-3)].¹⁶⁾ The assignment of the ^{13}C -NMR spectrum was based on comparison with data for ABTS and carbon-proton heteronuclear 2D chemical shift correlation *via* one-bond and long range J_{CH} .¹⁷⁾ Since the two signals corresponding to the carbon-nitrogen double bond appeared at 152.32 (C-2') and 171.80 (C-6) in the ^{13}C -NMR spectrum, compound I was concluded to have a structure in which 3-ethylbenzothiazoline-6-sulfonic acid and 2-carboxy-1,4-benzoquinone were linked at the 2-position of the former and the 1-

position of the latter by an azino group, and it was named the 6-(6'-sulfo-3'-ethylbenzothiazol-2'-ylidenehydrazono)-3-oxo-1,4-cyclohexadiene-1-carboxylic acid disodium salt.

Compound II, bluish black powder, mp $> 300^{\circ}\text{C}$, UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (log ϵ): 196 (4.44), 216 (4.39), 293 (3.85), 587 (4.24), was assigned the same molecular formula as that of I on the basis of the SIMS (MH^+ : m/z 452). The spectral data were very similar to those of I, but the signals of three protons of a benzoquinone moiety appeared at 6.25 (1H, d, $J=9.9$ Hz, 5-H), 7.65 (1H, dd, $J=9.9$, 2.1 Hz, 6-H) and 7.96 (1H, d, $J=2.1$ Hz, 2-H) in the ^1H -NMR spectrum ($\text{DMSO}-d_6$), and the ketone signal was shifted to 182.87 in the ^{13}C -NMR spectrum ($\text{DMSO}-d_6$). Based on these spectral data, the structure of II was concluded to be 3-(6'-sulfo-3'-ethylbenzothiazol-2'-ylidenehydrazono)-4-oxo-1,5'-cyclohexadiene-1-carboxylic acid disodium salt, which has a 1,2-benzoquinone moiety instead of the 1,4-benzoquinone moiety of I.

This is the first report of the isolation and structural determination of the product of an enzymatic colorimetric reaction using a coupling compound.

A more detailed description of this work will be presented later.

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