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Studies on the Antioxidants. XIV.[1] Reaction of Sesamol with Hydrogen Peroxide-Peroxidase

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Sesamol (I) was transformed into a fluorescent product (II) and a violet-colored product (IV) by mild oxidation with hydrogen peroxide catalyzed by horseradish peroxidase. The product (II) was elucidated to be 2,2′-dihydroxy-4,5; 4′,5′-bis(methylenedioxy)biphenyl and the structure of IV was assumed to be the highly conjugated quinoid form of II. The product (II) was transformed into IV by hydrogen peroxide-peroxidase treatment and was regenerated from IV by treatment with reductants. Systems containing II, hydrogen peroxide and peroxidase may provide a new, versatile chromogenic determination method for hydrogen peroxide or peroxidase. Determination of hydrogen peroxide at around 10 μM and peroxidase at around 0.1 nM was possible. Oxidations of I by other means were performed and the results were compared with those obtained with hydrogen peroxide-peroxidase.

Keywords——sesamol; hydrogen peroxide; horseradish peroxidase; chromogenic assay; 2,2′-dihydroxy-4,5; 4′5′-bis(methylenedioxy)biphenyl

Sesame oil, which is widely used as an edible oil and as a solvent for injections, contains three constituents: sesamin, sesamolin and sesamol.[2,3] Sesamol (3,4-methylenedioxyphenol, I) is formed from sesamolin during the processing of sesame oil and serves as an antioxidant to protect the oil from autoxidation. In the previous papers,[1,4-6] it was shown that the antioxidant (I) exhibited several characteristic properties in the redox system compared with other phenolic antioxidants. It seemed worthwhile to study how the antioxidant (I) is derivatized by oxidations. In the present work, mild oxidation of I with hydrogen peroxide-peroxidase was investigated and it was found that this system produced a fluorescent compound and a violet-colored compound. A study was then undertaken to determine how the system could be applied to the chromogenic assay of I, hydrogen peroxide and peroxidase.

Experimental

Materials——Sesamol (I) was a product of Aldrich Chemical Company, Inc. It was recrystallized from $CHCl_3$–petroleum ether for spectrophotometric determination. Commercial reagent-grade 31% H_2O_2 was estimated to be 9.73 M by iodometric titration. *t*-Butyl hydroperoxide (*t*BuOOH) (70%, 7.6 M, Nakarai Chemicals, Ltd.) was diluted with 6 volumes of dimethylsulfoxide for use. Horseradish peroxidase (HRPO) (Grade III, 100 units/mg, molecular weight: 40000) was a product of Toyobo Company, Ltd. Partially purified human oxyhemoglobin (HbO_2) and methemoglobin (MetHb) were prepared as described previously.[5,6] Catalase from bovine liver was a product of Sigma Chemical Company.

Methods——Thin layer chromatography (TLC) was performed on plates coated with Wako gel B-5F (Wako Pure Chemical Industries, Ltd.). Spots on a plate were detected by ultraviolet (UV) irradiation (254 and 365 nm) and by spraying a 1% solution of 2,4-dichloroquinone monochloroimide in EtOH (BQC reagent), which is specific for phenolics. Column chromatographies were performed on silica gel for chromatography (100 mesh, Kanto Chemical Company, Ltd.). Absorption spectra were measured with a Shimadzu UV-200S double beam spectrometer. Excitation and fluorescence spectra were measured with a Hitachi 204A fluorescence spectrophotometer. Mass spectra were obtained with a Hitachi RMU-7L double focusing mass spectrometer. Nuclear magnetic resonance (NMR) spectra were taken in d_6-dimethylsulfoxide with Me_4Si as an internal standard using a JEOL PS-100 machine.

The product (II) in reaction mixtures was determined by high performance liquid chromatography (HPLC) on a Shimadzu LC-2 liquid chromatograph with a stainless steel column (7.9 mm i.d. × 0.5 m) of Permaphase ODS. Elution was carried out with 10% EtOH at the flow rate of 1 ml/min. The peaks were detected at 314 nm and a range of 32 with a Shimadzu SPD-1 spectrophotometer. The $CHCl_3$ extract of the

reaction mixture of I and H_2O_2–HRPO was evaporated to dryness and the residue was redissolved in EtOH for injection, since direct injection of the extract disturbed the chromatogram. I was rapidly eluted together with the solvent and II was eluted later at a retention time of 3.1 min. Other products could not be detected under the conditions used. In every chromatography, the peak due to II was confirmed by a spectrophotometric scan. The amount of II was determined by comparison of the peak area with that of an authentic solution of II (4.38×10^{-4} M, 10 μl) in EtOH.

Reaction of I with Hydrogen Peroxide-HRPO——To a solution of 1.0 g (final: 69 mM) of I in 100 ml of water, 4.0 ml (final: 370 mM) of 31% H_2O_2 and 20 mg (final :5 μM) of HRPO were added. The solution immediately turned violet and afforded a precipitate. The mixture was kept at room temperature for 5 min, then diluted with 500 ml of water and extract with 300 ml of $CHCl_3$ three times. The combined extracts were subjected to TLC (Fig. 1). The extracts were evaporated to dryness *in vacuo* to obtain a gum. The gum was applied to a column of silica gel (60 g) and eluted with $CHCl_3$ to afford 330 mg of the product (II) and a small amount of the product (III). The product (II) was rechromatographed on a column of silica gel (15 g). The fractions containing II were evaporated to dryness to afford a crystalline powder, which was subsequently recrystallized from $CHCl_3$ to yield 147 mg (yield, 9.9%) of slightly violet-colored columns of II, decomposing at 202—208°C with shrinking at 175°C. TLC: *Rf* 0.26 ($CHCl_3$–EtOH, 39: 1), and 0.15 (*n*-hexane–AcOEt, 4: 1), single spot. NMR spectrum, ppm; 6.62 (2H, *s*, $H_{3,3'}$ or $H_{6,6'}$), 6.45 (2H, *s*, $H_{3,3'}$ or $H_{6,6'}$), 5.88 (4H, *s*, $-CH_2-$). MS, *m/z*: 274 (M^+). UV spectrum: λ_{max}^{EtOH} nm (ε), 314 (14600) and 251 (7300) Fig. 2). Fluorescence spectrum: (Fig. 3 and Table I). *Anal.* Calcd for $C_{14}H_{10}O_6$; C, 61.31; H. 3.67%. Found; C, 61.24; H, 3.64%.

Several attempts to purify or crystallize the minor fluorescent compound (III) were unsuccessful. The violet-colored product (IV) could not be detected in the eluates of the silica gel column, indicating that it had disappeared during the chromatography. The time-dependent decay of the product (IV) was monitored by measurement of the spectrum of the $CHCl_3$ extract of the reaction mixture. Thus, the extract was stored in a stoppered tube at room temperature for 22 hours. Absorption spectra of the extract showed that the violet-colored product (IV) with a maximum at around 530 nm gradually disappeared during storage (Fig. 4). Attempts to isolate or purify IV by a rapid dry column chromatography through silica gel or by collection of the precipitate from the reaction mixture were unsuccessful.

Results

Oxidation Products of Sesamol (I) formed by Reaction with Hydrogen Peroxide and Horseradish Peroxidase (HRPO)

Sesamol (I) was treated with a roughly 5-fold molar excess of hydrogen peroxide in the presence of horseradish peroxidase (HRPO). The solution immediately turned violet and afforded a precipitate. Most of the violet color and the precipitate were extracted with chloroform. TLC of the extract revealed three reaction products (II, III and IV) on a plate developed with a mixture of chloroform and ethanol and two products (II and III) on a plate developed with a mixture of *n*-hexane and ethyl acetate (Fig. 1). The fluorescent product (II) and the violet-colored product (IV) seemed to be the major products, but the latter could not always be detected, probably due to its instability.

The major fluorescent compound (II) was isolated by silica gel column chromatography as crystalline columns melting at 202—208 °C. The mass spectrum of II showed the molecular ion peak at *m/e* 274, suggesting that it was a dimeric product of I formed by loss of two hydrogen atoms from I. The NMR spectrum of I exhibited 3 aromatic protons as a double doublet at 6.16 ppm (H_6), a doublet at 6.35 ppm (H_2) and a doublet at 6.63 ppm (H_5) with coupling constants of $J_{2,6}=2$ Hz and $J_{5,6}=8$ Hz, and two methylene protons as a singlet at 5.84 ppm, while the spectrum of II showed two aromatic protons as singlets at 6.62 and 6.45 ppm and two methylene protons as a singlet at 5.88 ppm. Disappearance of a characteristic *meta* coupling of an aromatic ring is thus a strong indication that the dimerization has occurred at the 6 position of I. The structure 2,2′-dihydroxy-4,5;4′,5′-bis (methylenedioxy)biphenyl accounts for these spectra data of II. The UV absorption spectrum of II exhibited maxima at 314 nm and 251 nm, which were batho- and hyperchromically shifted from those of I at 295 and 233 nm.[7] (Fig. 2)

The fluorescence spectrum of II was compared with that of I (Fig. 3 and Table I). Sesamol (I) exhibited a weak fluorescence in the UV region with an excitation maximum (λex) at 300 nm

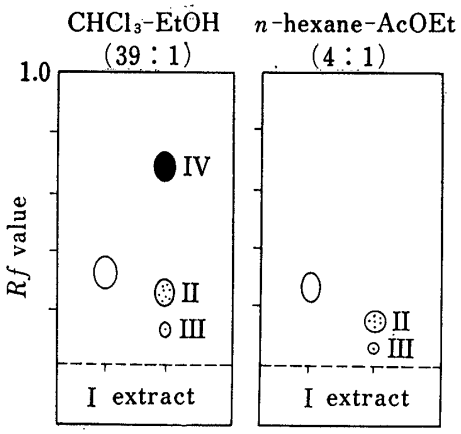

Fig. 1. TLC of the Chloroform Extract of the Reaction Mixture of I and Hydrogen Peroxide HRPO

Spots were visualized by violet coloration (●), UV absorption upon irradiation at 254 nm (○) and fluorescence upon excitation at 365 nm (◎). The spots corresponding to I, II and III were colored blue by spraying BQC reagent.

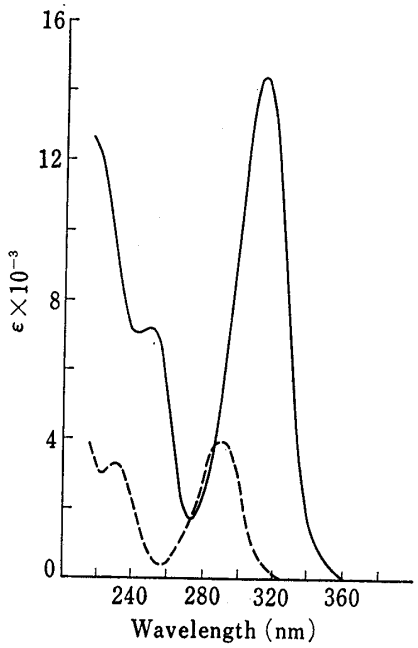

Fig. 2. Ultraviolet Absorption Spectra of I (----) and II (——)

and an emission maximum (λem) at 335 nm in water, ethanol and iso-butanol, but it was completely quenched in chloroform. The relative molar intensity with respect to quinine sulfate was less than 7%. The fluorescence of the dimeric product (II) in ethanol exhibited λex 311 and λem 373 nm with a relative molar intensity twice as large as that of quinine sulfate. The excitation and emission maxima of I shifted to longer wavelength by 11 and 38 nm, respectively, through *ortho-ortho* dimerization. The fluorescence intensity of II in iso-butanol was, however, half that in ethanol and it was much lower in water and chloroform. These fluorescence relationships between I and II were similar to those between butylated hydroxy-

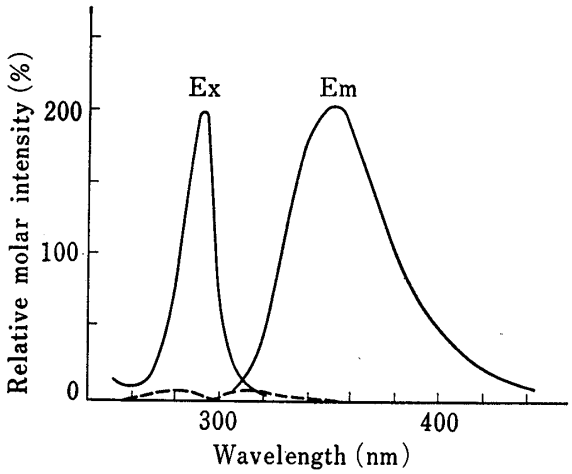

Fig. 3. Fluorescence Spectra of I (----) and II (——)

The spectra were taken with 1.14×10^{-5} M I (excitation at 300 nm and emission at 335 nm) and with 0.37×10^{-6}M II (excitation at 313 nm and emission at 373 nm) in ethanol. Relative molar intensity was calculated based on the fluorescence at 450 nm with excitation at 365 nm of 1×10^{-6} M quinine sulfate in 0.1 N H_2SO_4.

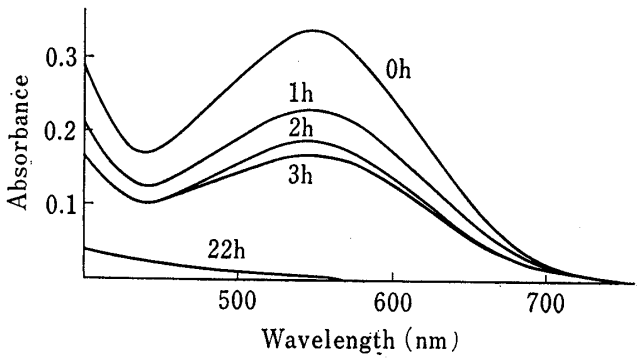

Fig. 4. Absorption Spectrum of the Chloroform Extract of the Reaction of I with Hydrogen Peroxide-HRPO

A mixture of 69 mM I, 370 mM H_2O_2 and 5 μM HRPO in treated for 5 min, then extracted with 10 volumes of $CHCl_3$. The spectra of the extract, kept at room temperature for the period indicated, were recorded after dilution into 40 volumes of EtOH.

Table I. Fluorescence Intensities of I and II

Compound	Solvent	Fluorescence maximum (nm)		Relative molar intensity	
		λex	λem	Ex (at nm)	Em (at nm)
Quinine sulfate[a]	0.1 N H_2SO_4	365	450	100(450)	100(365)
I[b]	H_2O	300	335	4.0(335)	3.7(300)
	EtOH	300	335	6.5(335)	6.5(300)
	iso-BuOH	300	335	6.4(335)	6.3(300)
	$CHCl_3$	—	—	—	—
II[c]	H_2O	311	380	4.2(373)	4.5(311)
	EtOH	311	373	200(373)	200(311)
	iso-BuOH	311	370	109(373)	100(311)
	$CHCl_3$	311	360	11(373)	10(311)

a) 1.12×10^{-6} M; *b*) 1.14 or 1.75×10^{-5} M; and *c*) 0.35×10^{-6} or 1.75×10^{-5} M.

anisole and its dimer, the former exhibiting λex 293 and λem 342[8] (or λex 296 and λem 324[9]) nm and the latter exhibiting λex 358 and λem 440 nm.[9] The *ortho-ortho* dimeric product of homovanillic acid also showed a strong fluorescence at λex 315 and λem 425 nm.[10]

The major violet-colored product (IV) could not be eluted from the silica gel column. The absorption spectrum of the chloroform extract exhibited a maximum at around 530 nm but the absorbance decreased with time (Fig. 4). The failure to isolate the product (IV) may be due to its lability during the work-up. Treatment of II with hydrogen peroxide-HRPO readily afforded IV, and I must have undergone oxidation to produce II which was in turn transformed into the highly oxidized violet-colored product (IV). Although there is no direct evidence on the structure of IV, IV might be the highly conjugated quinoid form of II produced by abstraction of two hydrogens.

Analysis of the Products (II and IV)

In order to characterize the reaction of I with hydrogen peroxide-HRPO, determination of the fluorescent product (II) and violet-colored product (IV) was attempted. The reaction produced a large amount of precipitate and it disturbed the direct assay. Extraction of the reaction mixture with chloroform was suitable, since II and IV were completely transferred and the enzyme was removed. The product (IV) was determined spectrophotometrically in terms of total absorbance at 530 nm. The product (II) could not be determined by a fluorometric assay owing to other fluorescent contaminants. The amount of II was thus determined by high performance liquid chromatography (HPLC) with a reverse phase column as shown in Fig. 5.

Mixtures of I and hydrogen peroxide-HRPO were treated at room temperature for 5 min, and the product analysis was carried out under identical work-up conditions (Fig. 6). When 48 mM I was treated with various amounts of hydrogen peroxide in the presence of a catalytic amount of HRPO, II was produced in a best yield of 25% at 26 mM hydrogen peroxide (Fig. 6A). The yield decreased to 9% at 42 mM hydrogen peroxide and gradually increased as the concentration of hydrogen peroxide increased. The absorbance due to IV reached a maximum at 42 mM hydrogen peroxide and gradually decreased with the hydrogen peroxide concentration. When 48 mM I was treated with 26 mM hydrogen peroxide in the presence of various amounts of HRPO, the yield of II increased with the enzyme concentration, while the absorbance due to IV reached a plateau at 1.6 μM HRPO (Fig. 6B). Prolonged treatment of these reaction mixtures (for several hours) did not improve the yield of these products, and the production of these compounds appeared to be instantaneous.

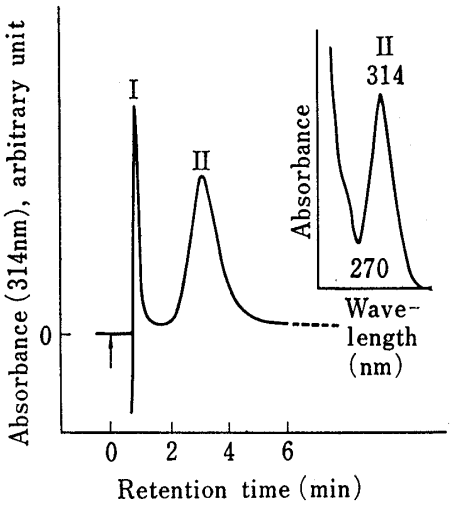

Fig. 5. HPLC of the Reaction Mixture of I and Hydrogen Peroxide-HRPO

A 1.50 ml mixture of 48 mM I (72 μmol), 250 mM H_2O_2 and 3.3 μM HRPO in water was kept for 5 min. It was diluted with 40 ml of water and extracted with 10 ml of $CHCl_3$. The extract was evaporated to dryness *in vacuo* below 30 °C and redissolved in an equal volume of EtOH for chromatography.

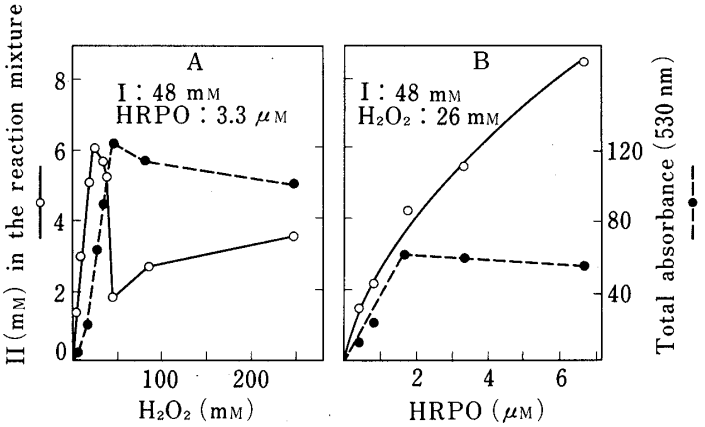

Fig. 6. Formation of II and IV in the Reaction of I with Hydrogen Peroxide-HRPO

A 1.50 ml mixture of I, H_2O_2-HRPO in water was treated for 5 min, then extracted with $CHCl_3$. Total absorbance was determined as soon as possible, and the amount of II was determined by HPLC as already described (Fig. 5).

The chloroform extract of the reaction mixture of I and hydrogen peroxide-HRPO was treated under various conditions (Table II). The absorbance of the extract due to IV decreased to about 50% upon evaporation and was lost completley upon heating with caustic compounds. A slight increase of II accompanied the complete loss of the absorbance on heating. Addition of large amounts of I to the extract caused spontaneous disappearance of the characteristic violet color and marked increses of II. The results may indicate that the loss of IV by heating induced the formation of II together with another colorless compound (s), while the loss of IV upon addition of I gave II in a higher yield. When II was treated with an excess of hydrogen peroxide-HRPO, the chloroform extract showed an absorption spectrum and a Rf value on TLC corresponding to those of IV (Table II). Concentration of the extract

Table II. Interconversion of II and IV

Treatment[a] of the $CHCl_3$ extract from	II(μmol)	IV (Total absorbance at 530 nm)
I and H_2O_2-HRPO[b]		
1) none		140
2) evaporation at 30 °C	5.0	72
3) boil for 5 min and evaporation	6.6	0
4) addition of I (50 mg) and evaporation	11.1	0
5) addition of I (150 mg) and evaporation	15.2	0
II and H_2O_2-HRPO[c]		
1) none[d]		5.5
2) evaporation at 30 °C	0.14	0
3) addition of I (30 mg) and evaporation	1.92	0
4) addition of *p*-hydroquinone (30 mg) and evaporation	1.90	0

a) After treatment, the extract was redissolved in an equal volume of EtOH for HPLC (Fig. 5).
b) I (72 μmol was treated as described in the legend to Fig. 5.
c) A 5.0 ml mixture of 0.4 mM II (2 μmol), 1 mM H_2O_2 and 4 μM HRPO in 10% EtOH was treated for 5 min.
d) The absorption spectrum of the $CHCl_3$ extract showed a maximum at 530 nm, and TLC ($CHCl_3$–EtOH, 39:1) revealed a single spot corresponding to **IV**.

caused loss of the absorbance and the amount of II was as little as 7% of the initial amount. When the extract was treated with a large excess of reductants such as I and *p*-hydroquinone, II was regenerated quantitatively (96% and 95%, respectively) with a complete loss of the absorbance. Thus, it may be concluded that I was transformed by hydrogen peroxide-HRPO into II which was in turn converted into IV, and that II was quantitatively regenerated from IV in the presence of a large excess of I. The complicated changes in amounts of II and IV (shown in Fig. 6) may be explained as the results of this sequence of reactions. The structure of IV may be the highly conjugated quinoid form of II shown in the chart.

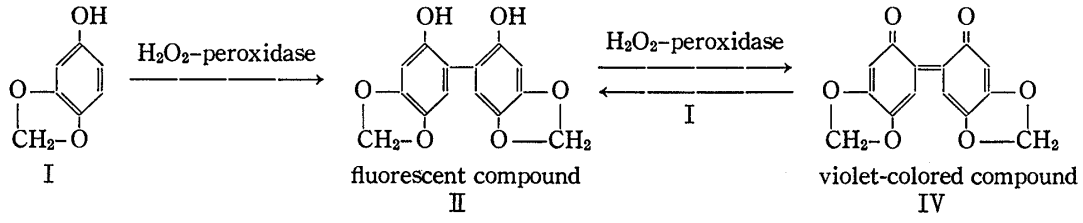

Chart 1

Application of the System Containing I (or II), Hydrogen Peroxide and HRPO to Chromogenic Assay for Each Reactant

The characteristic violet color produced by the reaction of I or II with hydrogen peroxide-HRPO was applied to the chromogenic assay of each reactant, and color developements at various low reactant concentrations were followed.

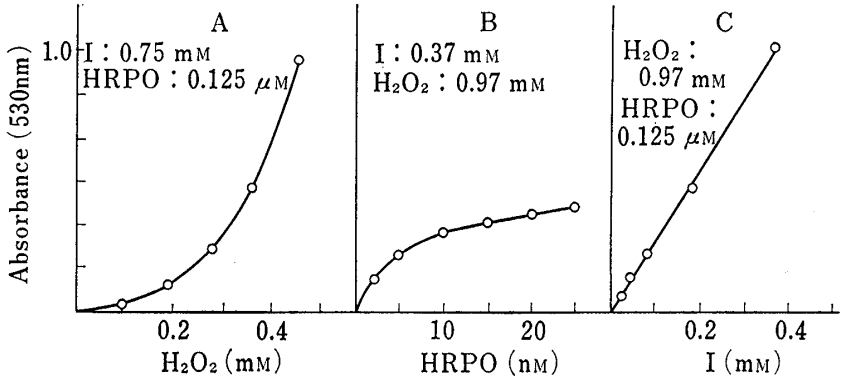

Fig. 7. Color Formation of a Mixture of I and Hydrogen Peroxide-HRPO

A solution of 3.50 ml of 0.1 M sodium phosphate buffer (pH 7.0) was mixed with 0.50 ml of H_2O_2 solution, 0.50 ml of I in water and 0.50 ml of HRPO solution. The mixture was kept for 10 min, then extracted with 5.0 ml of $CHCl_3$ for measurement of absorbance.

When various amounts of hydrogen peroxide were treated with an excess of I (Fig. 7A), the absorbance at 530 nm increased with the hydrogen peroxide concentration, but the relationship was not linear. The concave profile may be due to decolorization of IV by an excess of I. When I and hydrogen peroxide were treated with various amounts of HRPO (Fig. 7B), the color development increased with HRPO concentration, but not proportionally. When various amounts of I were treated with an excess of hydrogen peroxide, the color development increased proportionally with the concentration of I. Color developments in the systems were instantaneous but relatively unstable. The systems were not suitable for determination of each reactant owing to lack of linearity or stability of the color, though the systems appeared to be useful for the detection of each reactant. For example, the presence of I in sesame oil may be readily detected by the use of hydrogen peroxide and HRPO. A sesame oil preparation

which was colored in the Baudouin test,[2] sensitive to both I and sesamolin, was not colored by vigorous shaking with a mixture of 1 mM hydrogen peroxide and 1 μM HRPO, but the oil to which 10 μM had been added was intensely colored violet by shaking with the mixture. The minium concentrations required for visualization of the violet color were 10 μM for I, 100 μM for hydrogen peroxide and 1 nM for HRPO.

When various amounts of hydrogen peroxide were treated with II (Fig. 8A), the absorbance was rather stable over 2 hours and the plot of absorbance *vs.* hydrogen peroxide concentration was linear. When II and hydrogen peroxide were treated with various amounts of HRPO (Fig. 8), the color development increased linearly with respect to HRPO concentration at low levels. Thus a chromogenic assay for hydrogen peroxide and the peroxidase might be possible at low levels of about 10 μM hydrogen peroxide and 0.1 nM HRPO.

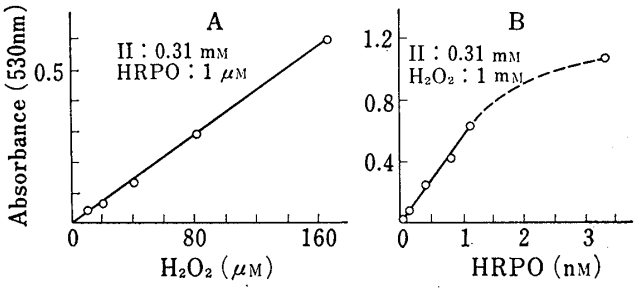

Fig. 8. Color Formation of a Mixture of II and Hydrogen Peroxide-HRPO

A solution of 3.50 ml of the phosphate buffer (pH 7.0) was mixed with 0.50 ml of II in EtOH and 0.50 ml of HRPO solution. The mixture was kept for 10 min, then extracted with 10 ml of $CHCl_3$ for measurement of absorbance.

Oxidations of I by Various Biochemical, Chemical and Physical Procedures

Several biochemical, chemical and physical oxidations of I were investigated and compared with that by hydrogen peroxide-HRPO. Treatment of I with hydrogen peroxide in the presence of hemoglobin (HbO_2 and MetHb) gave II and IV, but the overall yields were relatively low compared with those of the peroxidase-catalyzed reaction. Catalase was almost inert in combination with hydrogen peroxide. Treatment of I with *t*-BuOOH in the presence of HRPO gave only a little II.

Chemical oxidations of I with alkaline ferricyanide, alkaline ferric chloride and ferric chloride-α,α′-dipyridyl produced only trace amounts of II and IV, accompanied by many other unidentified products. Fenton's reagent did not afford II or IV.

UV irradiation of I gradually produced II. Thus, a solution of 10 mg of I in water gradually turned brown upon irradiation, and TLC of the mixture revealed one major fluorescent spot corresponding to II, the yields being 1.7% (42 hours) and 2.4% (90 hours) as estimated by HPLC. Silica gel effectively oxidized I into II. When 50 mg of I coated on 1.0 g of silica gel was heated at 100 °C for 1 hour, II was produced in a yield of 8.1% without any other product.

Discussion

A mild oxidation of sesamol (I), a monophenolic antioxidant in sesame oil, with hydrogen peroxide catalyzed by HRPO gave a fluorescent *ortho-ortho* dimeric product (II) and violet-colored product (IV) whose structure was assumed to be highly conjugated quinoid form of II. It has been demonstrated that homovanillic acid undergoes a similar type of reaction with hydrogen peroxide-HRPO to produce a fluorescent dimer.[11]

Hemoglobin also catalyzed the reaction of I with hydrogen peroxide to produce II and IV in lower yields. Caustic chemical oxidations of I with alkaline ferricyanide, alkaline ferric chloride and Emmerie-Engel reagent produced a complex mixture of products, the yields of II and IV being very small. The earlier observation that I donated 4 electrons to ferric ions in its reaction with Emmerie-Engel reagent[4] suggested that oxidations by ferric ions proceeded in preference to the formation of IV, which may require 2-electron abstraction from I. UV

irradiation or heating on silica gel transformed I into II, indicating that dimerization may take place during the processing or the storage of sesame oil.

The presence of I in sesame oil can be detected by coloration in the tests proposed by Villavecchia and Fabris, or by Bauduin,[2] but the tests are also positive to the bound form of sesamol (sesamolin).[12,13] The presence of free sesamol in sesame oil may be readily detected by coloration upon shaking with a solution of hydrogen peroxide-HRPO, while sesamolin does not give a coloration under the same conditions.

Before the discovery of the potent carcinogenic nature of benzidine, *o*-tolidine, *o*-toluidine and *o*-anisidine, those compounds were widely used as chromogens for the hydrogen peroxide-peroxidase reaction. Several less hazardous chromogen systems have since been developed, including oxidative coupling between aminoantipyrine-phenols,[14–16] aminoantipyrine-dimethylaniline,[17] and 3-methyl-2-benzothiazolinone hydrazone-dimethylaniline[18] (or 3-(dimethylamino) benzoic acid[19]).

Formation of characteristic violet-colored products (IV) by reaction of I or II with hydrogen peroxide-HRPO may be useful for the chromogenic detection of hydrogen peroxide and peroxidase. Determinations of hydrogen peroxide and peroxidase could be readily achieved with the system containing II, since it required only low concentrations of hydrogen peroxide (around 10 μM) and the peroxidase (around 0.1 nM) for the production of color, and the color was instantaneously produced and relatively stable. Concentrations of hydrogen peroxide detectable in these systems are comparable to those in the aminoantipyrine-phenol[15] or aminoantipyrine-3-(dimethylamino) benzoic acid[19] systems. Thus, we propose a new, sensitive, versatile and safe chromogenic assay for hydrogen peroxide and peroxidase.

The reaction of II with hydrogen peroxide-HRPO resembles that of gum guaiac resin. Since tincture of gum guaiac resin is turned blue by oxidizing agents, the resin is sometimes used for the detection of hydrogen peroxide and peroxidase. The active species in the gum was elucidated to be α-guaiaconic acid; it is turned into guaiacum blue, which is a highly conjugated bismethylenequinone.[20] Since gum guaiac resin contains many phenolics other than the substrate for the reaction, compound (II) may be superior to the gum for the detection or determination of both hydrogen peroxide and peroxidase.

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