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Fluorescent Derivatization of Aromatic Carboxylic Acids with Horseradish Peroxidase in the Presence of Excess Hydrogen Peroxide

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The fluorescent derivatization of aromatic carboxylic acids by the catalytic activity of horseradish peroxidase (HRP) in the presence of excess H_2O_2 was investigated. Four monocarboxylic acids, nine dicarboxylic acids, and two tricarboxylic acids, all of which are non- or weakly fluorescent, were effectively converted into fluorescent compounds using this new method. This technique was further developed for the fluorometric determination of trace amounts of terephthalic acid (**3c**) and lutidinic acid (**2b**), and linear calibration curves for concentrations between 2.5 and 20.0 nmol of terephthalic acid (**3c**) and 1.0 and 10.0 nmol of lutidinic acid (**2b**) were demonstrated. Compound III, an intermediate of HRP, played an essential role in this process. Additionally, lactoperoxidase and manganese peroxidase, peroxidases similar to HRP, showed successful fluorescent derivatization of nicotinic acid (**1b**), lutidinic acid (**2b**), and hemimellitic acid (**4a**) in the presence of excess H_2O_2.

Keywords Fluorescent derivatization, horseradish peroxidase, hydrogen peroxide, aromatic carboxylic acid, compound III

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Introduction

Horseradish peroxidase (HRP) catalyzes reaction (1), shown below, in which a wide variety of substrates (AH_2) are catalyzed by HRP in the presence of H_2O_2.[1,2] This catalytic activity is routinely applied to quantify trace amounts of H_2O_2 in various fields of chemistry. For example, HRP is routinely utilized to measure H_2O_2 produced as a by-product of the action of oxidases on key compounds in clinical analysis, including glucose, uric acid, and cholesterol.[3]

$$AH_2 + H_2O_2 \xrightarrow{HRP} A + 2H_2O \quad (1)$$

When the amount of H_2O_2 is lower than that of AH_2, reaction (1) proceeds quantitatively. Conversely, we have previously tried to apply the catalytic activity of HRP to the fluorometric determination of trace amounts of AH_2, and have demonstrated that non-fluorescent tryptophan metabolites, such as nicotinic acid (**1b**), quinolinic acid (**2a**), kynurenic acid (**8a**), and xanthurenic acid (**8b**) (Fig. 1), all of which are aromatic carboxylic acids, are converted fluorescent compounds by the catalytic activity of HRP in the presence of excess H_2O_2.[4-6] In this study, we further investigated the action of HRP in the presence of excess H_2O_2 on various aromatic carboxylic acids shown in Fig. 1 to determine whether such activity, as previously demonstrated, would yield fluorescent compounds. Additionally, we investigated the potential of lactoperoxidase (LPO) and manganese peroxidase (MnP) as substitutes for HRP in this system.

The HRP enzyme intermediates known as compounds I and II are well known to play an important role in reaction (1) in the presence of small amounts of H_2O_2.[7,8] However, in the presence of excess H_2O_2, other HRP intermediates have been observed such as compound III and P-670.[9-12] It would therefore be interesting to determine which HRP intermediates participate in the fluorescent derivatization of the aforementioned compounds, which we herein attempt to ascertain by investigating the UV spectra of various reaction solutions.

Aromatic carboxylic acids are used in great quantity in various fields such as industrial and food chemistry. For example, in industrial chemistry, isophthalic acid (**3b**) and terephthalic acid (**3c**) are used in the production of synthetic fibers, while phthalic acid (**3a**) and trimellitic acid (**4b**) are used in the production of synthetic resins.[13,14] In food chemistry, benzoic acid (**1d**) is used as a preservative in cold beverages.[15,16] Moreover, dipicolinic acid (**2d**) comprises about 10% of the dry weight of spores of some members of the *Bacillus* genus, such as *Bacillus subtilis* var. *natto* (one of the representative useful bacteria),[17] and is therefore present in many common foods such as fermented soybean (natto). Kynurenic acid (**8a**), xanthurenic acid (**8b**), nicotinic acid (**1b**), and quinolinic acid (**2a**) are all well-known tryptophan metabolites of interest given their connection with various diseases,[18-20] while nicotinic acid (**1b**), picolinic acid (**1a**), and their derivatives show potential antimicrobial and antitumor activity.[21-23] Therefore, fluorescent derivatization of these non-fluorescent compounds with HRP may lead to new fluorometric quantification methods with a wide variety of applications.

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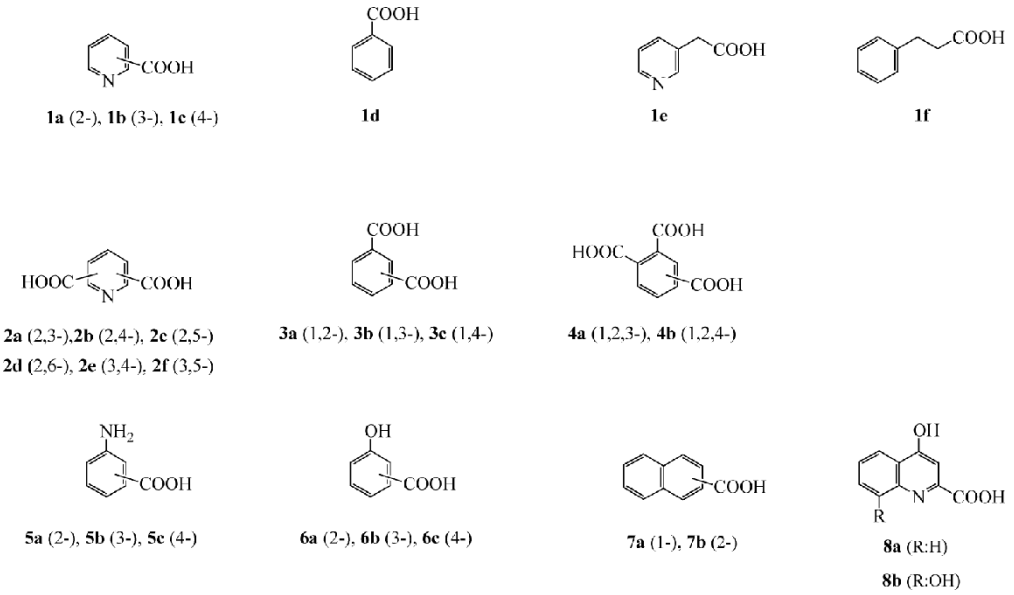

Fig. 1 Structures of the aromatic carboxylic acids used in this study. The position of the carboxyl group(s) is shown in parentheses.

Experimental

Reagents and apparatus

HRP (EC 1.11.1.7, type VI, activity: 100 units mg^{-1}) was purchased from Wako Pure Chemical Industries Co. (Osaka, Japan). LPO (EC 1.11.1.7, from bovine milk, in ammonium sulfate suspension, activity: ≥50 units mg^{-1}) and MnP (EC 1.11.1.13, from *Phanerochaete chrysosporium*, activity: ≥10 units mg^{-1}) were purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals were of analytical or reagent grade and used without further purification. HRP and MnP solutions were prepared by dissolving the purchased HRP and MnP powders in water, respectively. LPO solutions were prepared by diluting the purchased LPO solution with water. In this study, all peroxidase solutions were used within two weeks of preparation, and were stored in a refrigerator until use.

Fluorescence spectra were recorded on a Shimadzu RF-5300 spectrofluorometer with a quartz cell (1 cm × 1 cm cross-section) equipped with a xenon lamp and dual monochromator. For all experiments in this study, the bandwidths for both excitation and emission were set to 10 nm, unless otherwise noted.

Fluorescent derivatization with HRP in the presence of excess H_2O_2

The HRP solution (1.0 mL, 10 units mL^{-1}) was added to a mixture containing a sample solution (1.0 mL), 0.5 M H_2O_2 (1.0 mL), and 0.1 M lactate buffer with pH 5.0 (3.0 mL); the mixture was incubated at 35°C for 60 min in darkness. Both fluorescence excitation and emission wavelengths were measured for each substrate. The fluorescence intensity of blank solutions was measured under the same conditions. All experiments were carried out in darkness to avoid any photochemical reactions that could result in undesired fluorescent derivatization.

Results and Discussion

Fluorescent derivatization of non- or weakly fluorescent aromatic carboxylic acids with HRP

Previously, we demonstrated that non-fluorescent tryptophan metabolites, such as nicotinic acid (**1b**), quinolinic acid (**2a**), kynurenic acid (**8a**), and xanthurenic acid (**8b**), were converted into fluorescent compounds by the catalytic activity of HRP in the presence of excess H_2O_2.[4-6] So, the fluorescent derivatization of various non- or weakly fluorescent aromatic carboxylic acids in Fig. 1 was investigated in the presence of excess H_2O_2. Figure 2 shows the excitation and emission spectra of reaction solutions containing a non- or weakly fluorescent aromatic carboxylic acid, HRP, and H_2O_2. Non-fluorescent isonicotinic acid (**1c**) was converted into a fluorescent compound with excitation and emission wavelengths of 307 and 405 nm, respectively; however, solutions without HRP and/or H_2O_2 showed no or almost no fluorescence under otherwise identical conditions. The weak fluorescent intensity of the reaction solution containing only HRP was measured to have excitation and emission wavelengths of 290 and 440 nm, respectively, as shown in spectra (b) of Fig. 2(A); these excitation and emission wavelengths likely derive from very highly fluorescent tryptophan residues in HRP. Additionally, non-fluorescent terephthalic acid (**3c**), lutidinic acid (**2b**), and hemimellitic acid (**4a**) were converted into fluorescent compounds with excitation and emission wavelengths of 309 and 431 nm, 322 and 401 nm, and 300 and 429 nm, respectively. Accordingly, it was apparent that these substrates were converted into a fluorescent compound by the catalytic activity of HRP in the presence of excess H_2O_2. The results obtained for other non- or weakly fluorescent aromatic carboxylic acids are shown in Table 1. Except for only 3-pyridylacetic acid (**1e**) and 3-phenylpropionic acid (**1f**), all the other compounds were converted into fluorescent compounds. Interestingly, the excitation and emission wavelengths observed for each substrate were different, indicating that each substrate was converted into a different compound. This feature of the developed technique makes it particularly useful in analyzing

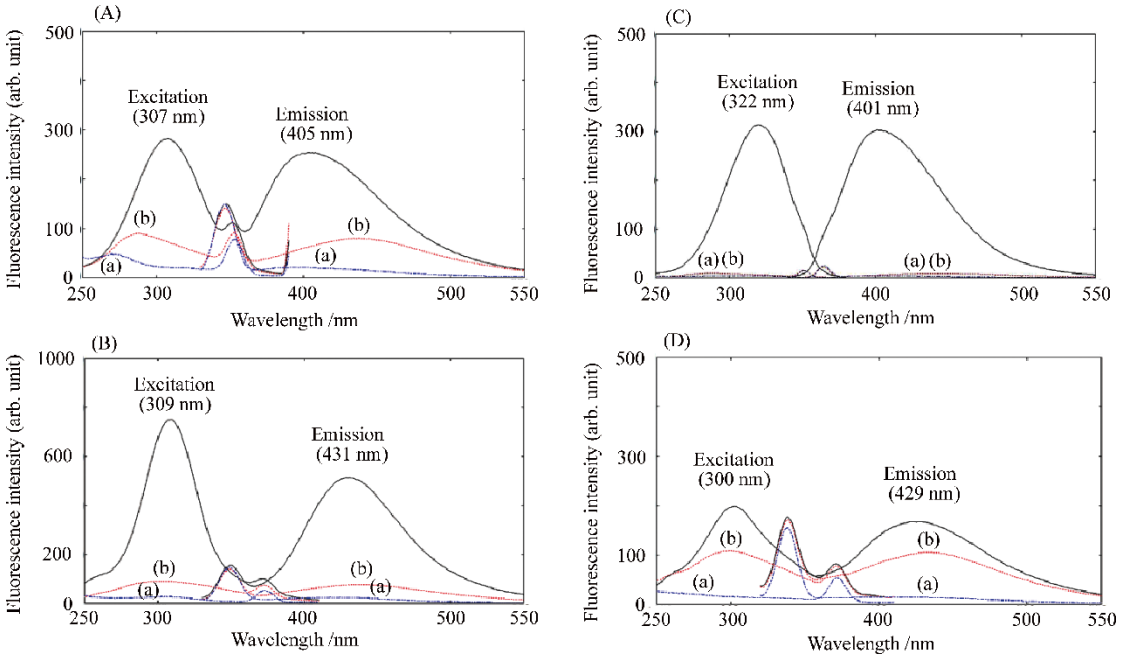

Fig. 2 Excitation and emission spectra of reaction solutions containing a non- or weakly fluorescent substrate before and after incubation with HRP in the presence of excess H_2O_2. After a mixture containing a substrate solution (1.0 mL, 20 nmol), 0.5 M H_2O_2 solution (1.0 mL), 10 units mL^{-1} HRP solution (1.0 mL) and 0.1 M lactate buffer solution (3.0 mL, pH 5.0) was incubated at 35°C for 60 min, the fluorescence intensity of the mixture was measured. Spectra (a) and (b) show the fluorescence of the substrate and that of the respective blank, respectively. (A): isonicotinic acid (**1c**); (B): terephthalic acid (**3c**); (C): lutidinic acid (**2b**; bandwidths: 5 nm); (D): hemimellitic acid (**4a**).

Table 1 Fluorescent derivatization of non- or weakly fluorescent substrates with HRP in the presence of excess H_2O_2

	Substrates	Common name	Ex /nm	Em /nm	Peak intensity at Em wavelength/nm
Monocyclic monocarboxylic acids	**1a**	Picolinic acid	307	394	137
	1b	Nicotinic acid	318	380	100
	1c	Isonicotinic acid	307	405	190
	1d	Benzoic acid	296	415	90
	1e	3-Pyridylacetic acid	—	—	—
	1f	3-Phenylpropionic acid	—	—	—
Monocyclic dicarboxylic acids	**2a**	Quinolinic acid	317	382	513
	2b	Lutidinic acid	322	401	284[a]
	2c	Isocinchomeronic acid	310	404	354
	2d	Dipicolinic acid	297	403	528
	2e	Cinchomeronic acid	306	427	125
	2f	Dinicotinic acid	316	434	45
	3a	Phthalic acid	300	425	95
	3b	Isophthalic acid	304	423	151
	3c	Terephthalic acid	309	431	440
Monocyclic tricarboxylic acids	**4a**	Hemimellitic acid	300	429	65
	4b	Trimellitic acid	303	438	97
Dicyclic monocarboxylic acids	**8a**	Kynurenic acid	367	470	390
	8b	Xanthurenic acid	325	435	720

a. The bandwidths were set at 5 nm for both excitation and emission.

and identifying different compounds. Considering that the compounds that were not converted into fluorescent compounds lack a carboxyl group directly bound to an aromatic ring, it is likely that only compounds containing this feature can be analyzed using this technique.

In the catalytic reaction (1), it is well known that AH_2 acts as a proton donor. For example, non-fluorescent 3-(*p*-hydroxyphenyl)propionic acid and homovanillic acid have been shown to donate a proton and then to be oxidized into a fluorescent dimer.[24,25] This may also occur with the tested substrates, though the structure of the reaction products remains unclear. In particular, certain substrates such as hemimellitic

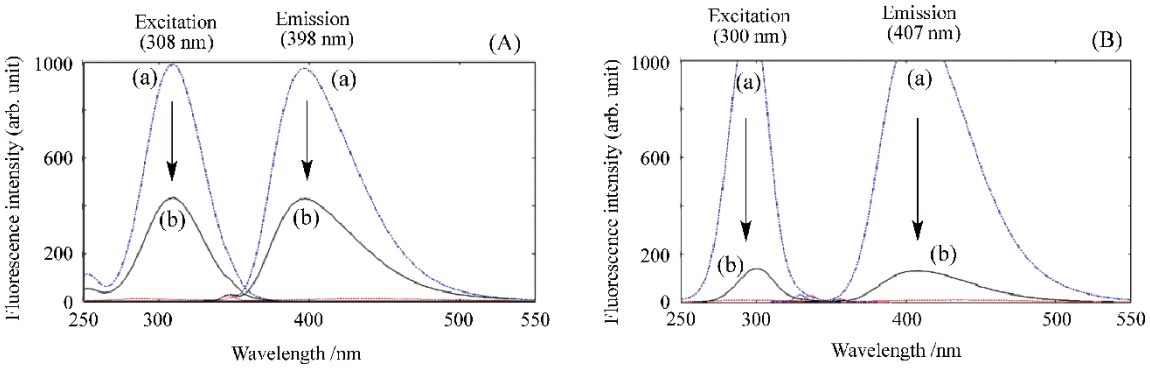

Fig. 3 Excitation and emission spectra of reaction solutions containing a fluorescent substrate before and after incubation with HRP in the presence of excess H_2O_2. After a mixture containing a substrate solution (1.0 mL, 20 nmol), 0.5 M H_2O_2 solution (1.0 mL), 10 units mL^{-1} HRP solution (1.0 mL) and 0.1 M lactate buffer solution (3.0 mL, pH 5.0) was incubated at 35°C for 60 min, the fluorescence intensity of the mixture was measured. Lines (a) and (b) show the spectra taken before and after incubation, respectively. (A): anthranilic acid (**5a**; bandwidths: 5 nm); (B): salicylic acid (**6a**; bandwidths: 5 nm).

Table 2 Effect of the catalytic activity of HRP for fluorescent substrates with HRP in the presence of excess H_2O_2

	Substrates	Common name	Ex /nm	Em /nm	Peak intensity change at Em wavelength (nm) before and after incubation (before → after)
Monocyclic monocarboxylic acids	**5a**	Anthranilic acid	308	398	970 → 474
	5b	*m*-Aminobenzoic acid	298	405	315 → 236
	5c	*p*-Aminobenzoic acid	275	339	505 → 417
	6a	Salicylic acid	300	407	>1000 → 130
	6b	*m*-Hydroxybenzoic acid	286	409	417 → 325
	6c	*p*-Hydroxybenzoic acid	297	431	327 → 315
Dicyclic monocarboxylic acids	**7a**	1-Naphthoic acid	281	374	138 → 126
	7b	2-Naphthoic acid	279	357	296 → 280

acid (**4a**) and trimellitic acid (**4b**) are more unlikely to form these dimers due to the steric hindrance introduced by multiple carboxylic acid groups.

Alternatively, other substituent effects may account for the increased fluorescence. While these effects are quite complex, it is empirically known that the presence of an electron-withdrawing group (–COOH, $–NO_2$, and $–CH_2COOH$, and so on) tends to decrease fluorescent activity, while the presence of an electron-donating group (–OH, $–NH_2$, and their alkyl derivatives) increases it.[26] This information suggests that it is the introduction of a hydroxyl group to the tested substrates that leads to the observed fluorescent activity.

Reactivity of HRP toward fluorescent aromatic carboxylic acids

The developed method was also applied to fluorescent aromatic carboxylic acids in order to investigate whether such compounds would either be converted into other fluorescent compounds or else non-fluorescent compounds. Figure 3 shows the excitation and emission spectra of the reaction solutions containing anthranilic acid (**5a**) or salicylic acid (**6a**), HRP, and H_2O_2 obtained before and after incubation. As these compounds were very highly fluorescent, the bandwidths for both excitation and emission were set to 5 nm for all experiments. The maximum excitation and emission wavelengths of anthranilic acid (**5a**) were observed at 308 and 398 nm, while those of salicylic acid (**6a**) were observed at 300 and 407 nm, respectively. As shown in Fig. 3, a decrease in intensity for both excitation and emission was observed for both samples after incubation, while no other fluorescent peaks were observed. These changes demonstrate that the tested compounds were oxidized to non-fluorescent compounds by HRP. The results obtained for other fluorescent aromatic carboxylic acids are shown in Table 2; of these compounds, *p*-hydroxybenzoic acid (**6c**), 1-naphthoic acid (**7a**), and 2-naphthoic acid (**7b**) were especially stable and showed almost no change in excitation and emission.

*Application to the fluorometric determination of terephthalic acid (**3c**) and lutidinic acid (**2b**)*

Terephthalic acid (**3c**) and lutidinic acid (**2b**) were tested by using the developed fluorescent derivatization method in order to evaluate the extent to which the technique proceeds quantitatively for non-fluorescent aromatic carboxylic acids. In order to establish the fluorometric determination of terephthalic acid (**3c**) and lutidinic acid (**2b**), the effects of various factors, such as pH, the concentrations of H_2O_2 and HRP, and the incubation time and temperature, on the fluorescent derivatization of each substrate with HRP were investigated. In both cases, the highest and constant fluorescence intensities were obtained in buffer solutions of pH 5.0 – 6.0, in the range of 0.5 – 1.0 M of H_2O_2, over 20 units mL^{-1} of HRP, above 60-min incubation time, and between 30 and 40°C of the incubation temperature. Based on these optimum conditions, samples were prepared by combining a substrate solution (1.0 mL) of variable concentration

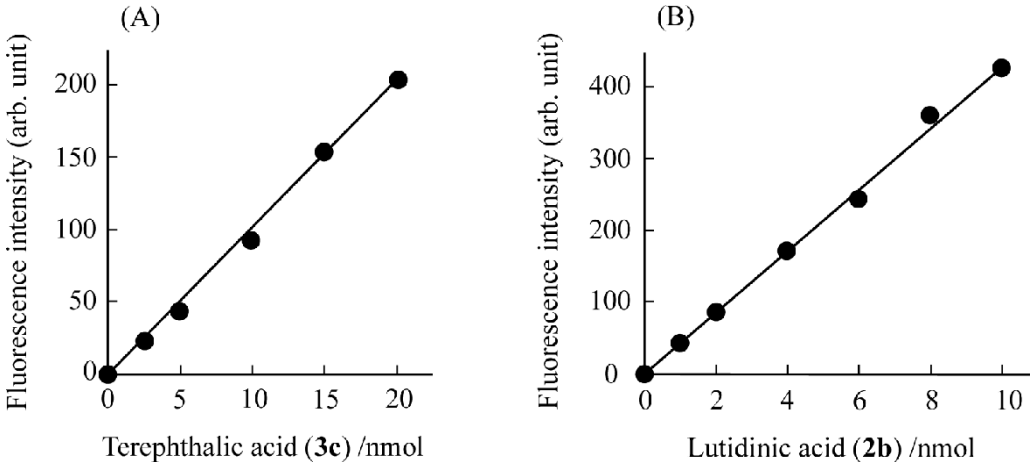

Fig. 4 Calibration curves for substrate reaction with HRP in the presence of excess H_2O_2. (A): terephthalic acid (**3c**); (B): lutidinic acid (**2b**).

with the HRP solution (1.0 mL, 20 units mL^{-1}), 0.5 M H_2O_2 (1.0 mL), and 0.1 M lactate buffer with pH 5.0 (3.0 mL); the mixture was incubated at 35°C for 60 min in darkness.

In the case of terephthalic acid (**3c**), the fluorescence intensity was measured for excitation and emission wavelengths of 309 and 431 nm, respectively, while the reagent blank solution was measured under similar conditions. As shown in Fig. 4(A), a linear calibration curve was obtained for terephthalic acid (**3c**) at concentrations between 2.5 and 20.0 nmol, with a calculated correlation coefficient and relative standard deviation (n = 5) of 0.989 and 4.9% for a concentration of 10.0 nmol, respectively. The limit of detection (LOD) and qualification (LOQ) were calculated as 3 and 10-times the standard deviation of the reagent blank (n = 10) divided by the sensitivity (the slope of the calibration curve), and were estimated at 0.46 and 1.5 nmol, respectively. The fluorescent derivative was stable because the fluorescence intensity of the reaction solution was almost constant for at least 2 h.

Similarly to terephthalic acid (**3c**), the fluorescence intensity of the mixture solution containing lutidinic acid (**2b**) was measured with excitation and emission wavelengths of 322 and 401 nm, respectively. Meanwhile, Fig. 4(B) shows that a linear calibration curve was also obtained for lutidinic acid (**2b**) with concentrations between 1.0 and 10.0 nmol. The correlation coefficient and relative standard deviation (n = 5) were 0.991 and 3.8% for a concentration of 5.0 nmol, respectively. LOD and LOQ were 0.10 and 0.32 nmol, respectively. The fluorescent derivative in reaction solutions was stable for at least 2 h. Terephthalic acid (**3c**) is used in the production of synthetic fibers,[27] while lutidinic acid (**2b**) plays a physiological role in plant growth, making the development of this technique worthwhile.[28]

Interference for the determination of each substrate was investigated in the presence of various foreign substances. In both cases, ascorbic acid, iodide ion, and human serum albumin (HSA) caused marked interference in increasing amounts. Other ions and substances, such as heparin, glycine, citric acid, acetic acid, NH_4^+, Fe^{3+}, Ca^{2+}, CO_3^{2-}, Br^-, and F^-, showed almost no interference even in the presence of 10-times the amount of each substrate.

Investigation of UV spectra of reaction solutions

In the presence of small amounts of H_2O_2, HRP is well known to catalyze reaction (1), in which active compounds I and II as transient intermediates of HRP play an important role.[7,8] However, in the presence of excess H_2O_2, other forms, such as compound III and P-670, are reported to be additionally formed, except for compounds I and II.[9–12] The UV spectra of the reaction solution of lutidinic acid (**2b**) were continuously collected during the course of fluorescent derivatization to determine the HRP intermediates that contribute to the conversion. Figure 5 shows the UV spectra of a sample solution containing only HRP before and after the addition of excess H_2O_2. The spectrum taken before the addition of H_2O_2 reveals the characteristic bands of the native form of HRP at 404, 498, and 639 nm while that taken after reveals the characteristic bands of compound III at 417, 543, and 577 nm, and a very weak characteristic band of P-670 at 669 nm. The characteristic peaks of compounds I and II, expected at 404, 550, and 650 nm and at 420, 527, and 556 nm, respectively, were not observed.[9]

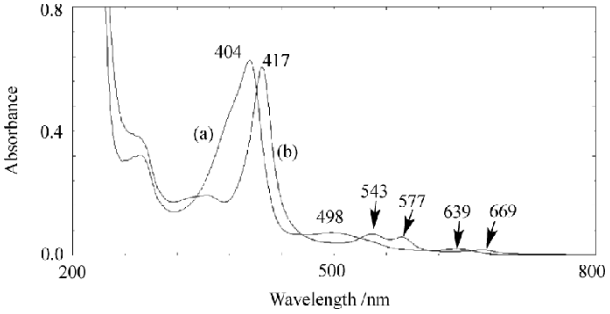

Fig. 5 UV absorption spectra of HRP solution before and after addition of excess H_2O_2. Spectra (a) and (b) were observed before and after the addition of 0.5 M H_2O_2 solution (1.0 mL) to a mixture of 100 units mL^{-1} HRP solution (1.0 mL) and 0.1 M lactate buffer solution (3.0 mL, pH 5).

The spectrum further changed upon the addition of lutidinic acid (**2b**), as shown in Fig. 6(A). The relative intensities of the characteristic bands of compound III at 417, 543, and 578 nm decreased along with an increase in that of the characteristic band of P-670 at around 670 nm. As shown in Fig. 6(B), the absorption spectra crossed at a point near 600 nm, although this point was not necessarily found to be isosbestic. These spectral changes indicate that compound III was converted to P-670

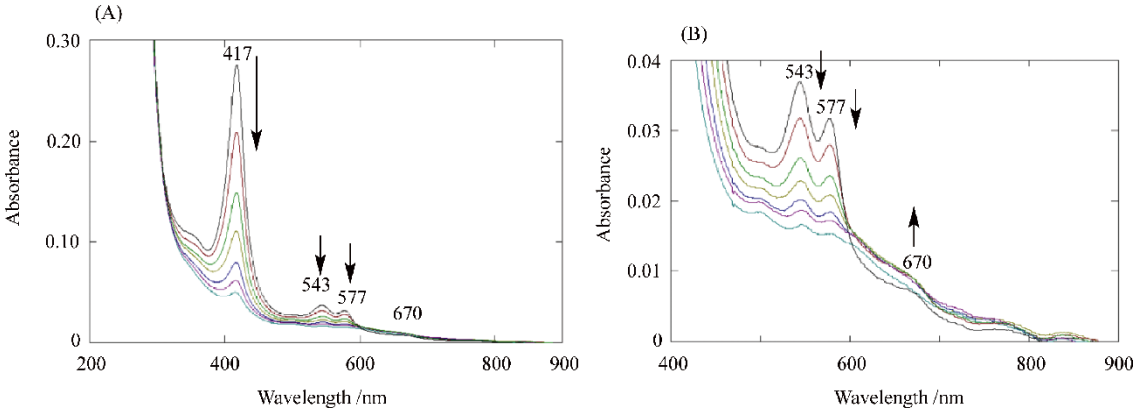

Fig. 6 UV absorption spectra of a reaction solution after the addition of lutidinic acid (**2b**). The spectra were periodically observed for up to 30 min after the addition of a solution of lutidinic acid (**2b**) (1.0 mL, 20 nmol) to a mixture of 100 units mL^{-1} HRP solution (1.0 mL), 0.5 M H_2O_2 solution (1.0 mL), and 0.1 M lactate buffer solution with pH 5.0 (3.0 mL). The arrow indicates the direction of absorption change with time. (A): observed at approximately 7 min intervals; (B): observed at approximately 4 min intervals.

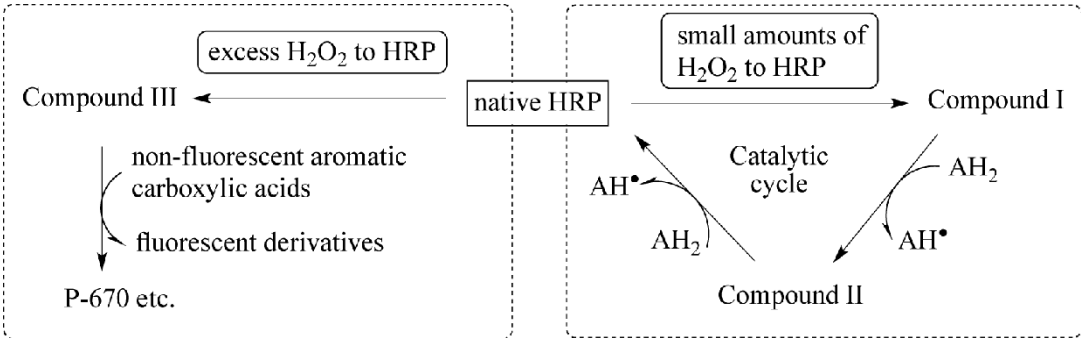

Fig. 7 Proposed reaction mechanisms of HRP-mediated fluorescent derivatization.

meaning that compound III was the enzyme intermediate that dominated. Accordingly, compound III likely plays a similar role in the reaction of other carboxylic acids as well, as shown in Fig. 7.

Investigation with LPO and MnP

HRP, LPO, and MnP catalyze the oxidation of inorganic and/or organic substrates with H_2O_2.[29] Similarly to HRP, the fluorescent derivatization with LPO or MnP was investigated in the presence of excess H_2O_2. Figure 8 shows the excitation and emission spectra for the fluorescent derivatization of lutidinic acid (**2b**) with LPO and MnP. In both cases, lutidinic acid (**2b**) was converted into a fluorescent compound similar to the one produced using HRP, as demonstrated by the excitation and emission peaks observed at 322 and 411 nm in the LPO spectrum and 322 and 414 nm in the MnP spectrum, respectively; these values correlate nicely with the observed HRP peaks at 322 and 401 nm for excitation and emission, respectively. Similar results were observed for nicotinic acid (**1b**) and hemimellitic acid (**4a**), as shown in Table 3.

Compounds I and II have been shown to be important transient intermediates in conventional LPO and MnP oxidation for catalytic amounts of H_2O_2 in much the same way that they are for HRP.[30,31] Additionally, it has been demonstrated that compound III is also produced in the case of LPO and MnP fluorescent derivatization with excess H_2O_2, although it is less active than compounds I and II.[32,33] Because of the low activity of compound III,[34] fluorescent derivatization with HRP, LPO, and MnP using the conditions developed in this study will likely require a longer incubation time.

Conclusions

Non- or weakly fluorescent aromatic carboxylic acids, with the exception of 3-pyridylacetic acid (**1e**) and 3-phenylpropionic acid (**1f**), were converted into the corresponding fluorescent compounds by the catalytic activity of HRP in the presence of excess H_2O_2. This fluorescent derivatization technique was then used to determine the presence of trace amounts of non-fluorescent terephthalic acid (**3c**) and lutidinic acid (**2b**). This new technique will likely be useful in converting non- or weakly fluorescent aromatic carboxylic acids into fluorescent compounds. Finally, data suggest that compound III, an intermediate of HRP, played an essential role in this new fluorescent derivatization process.

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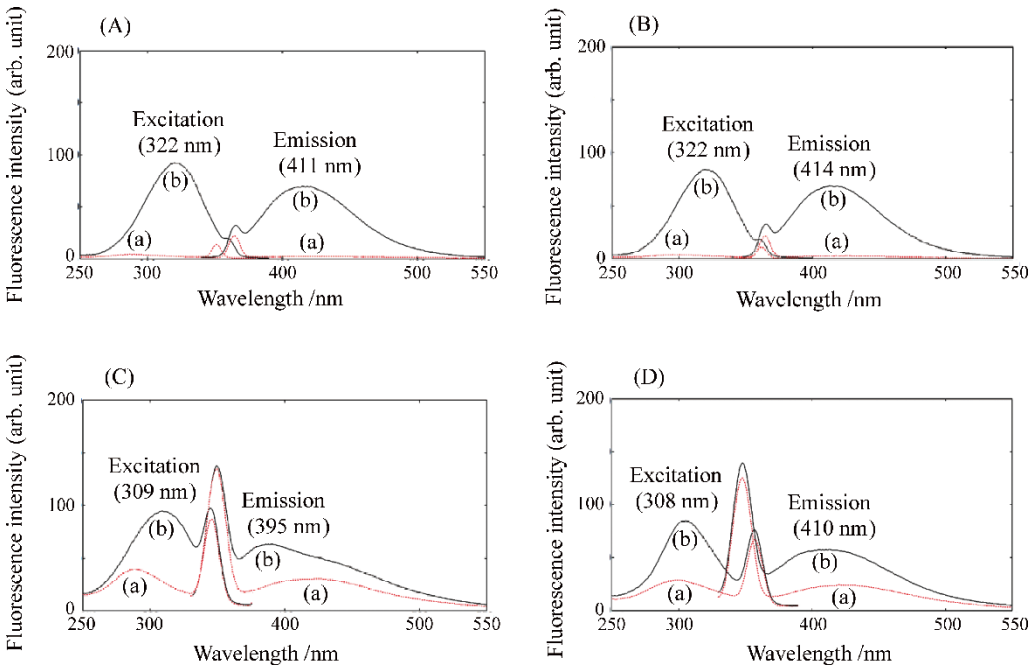

Fig. 8 Excitation and emission spectra of reaction solution containing lutidinic acid (**2b**) and hemimellitic acid (**4a**) before and after incubation with LPO or MnP in the presence of excess H_2O_2; Lines (a) and (b) show the spectrum before and after incubation, respectively. (A): lutidinic acid (**2b**) with LPO (bandwidths: 5 nm); (B): lutidinic acid (**2b**) with MnP (bandwidths: 5 nm); (C): hemimellitic acid (**4a**) with LPO; (D): hemimellitic acid (**4a**) with MnP.

Table 3 Effect of peroxidases on fluorescent derivatization of various substrates in the presence of excess H_2O_2

Substrates	POD	Ex	Em
		/nm	/nm
Nicotinic acid (**1b**)	HRP	318	380
	LPO	288	372
	MnP	312	391
Lutidinic acid (**2b**)	HRP	322	401
	LPO	322	414
	MnP	322	415
Hemimellitic acid (**4a**)	HRP	300	429
	LPO	309	395
	MnP	308	410

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