Fluorometric Determination of Quinolinic Acid Using the Catalytic Activity of Horseradish Peroxidase

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The conversion of quinolinic acid (QA) into a fluorescent compound by the catalytic activity of horseradish peroxidase (HRP) was investigated in the presence of hydrogen peroxide without exposure to light. Nonfluorescent quinolinic acid was converted into a fluorescent compound with maximum excitation and emission wavelengths at 328 and 377 nm, respectively. This fluorescent derivatization reaction with HRP in the presence of hydrogen peroxide was adopted for the determination of trace amounts of QA. The calibration curve obtained was linear from 0.1 to 5.0 nmol of QA in a 1.0 ml sample solution. The detection limit was 0.04 nmol/ml. The relative standard deviation at 3.0 nmol of QA was 3.58% (n = 8). This method was applied to the determination of QA spiked in control serum I and II. The recovery rates of QA were greater than 98%, and satisfactory results were obtained for both control serum I and II.

Key words —— quinolinic acid, horseradish peroxidase, catalytic activity, fluorescent derivatization, fluorometric determination, tryptophan metabolite

INTRODUCTION

Amino acids and their metabolites in vital fluids are well known to be related to inborn errors in amino acid metabolism1,2 and acquired diseases,3–5) Quinolinic acid (QA), one of the tryptophan metabolites, is of great interest in terms of its connection with some diseases, such as malaria, hepatic injury, Alzheimer’s disease, and Huntington’s disease.6–11) Thus it is important to measure trace amounts of QA in vital fluids,12–14) such as blood, urine, and cerebrospinal fluid, for proper diagnosis of such diseases. Various methods have been used for measuring trace amounts of QA in vital fluids, including photometric and fluorometric determination, etc.12,15–21) In particular, Mawatari et al. demonstrated a sensitive fluorometric method using the conversion of QA into a fluorescent compound in a photochemical reaction through UV irradiation in the presence of H2O2.17) Although the reported methods have been successfully applied to the determination of trace amounts of QA in vital fluids, some disadvantages still remain such as tedious procedures and/or lack of sensitivity.

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In a previous study,22) we attempted to determine whether the catalytic activity of horseradish peroxidase (HRP) in the presence of excess H2O2 was usable for the fluorescent derivatization of a nonfluorescent substrate (AH2) into a fluorescent compound according to the following reaction (1), which is routinely applied in the determination of trace amounts of H2O2 in clinical and environmental analyses.23,24)

\[ \text{AH}_2 + \text{H}_2\text{O}_2 \xrightarrow{\text{HRP}} \text{A} + 2\text{H}_2\text{O} \] (1)

As a result, we demonstrated that nonfluorescent kynurenic acid, one of the tryptophan metabolites, was converted into a fluorescent compound by HRP in the presence of excess H2O2 without exposure to light, and this fluorescent derivatization was applied to the determination of trace amounts of kynurenic acid spiked in control sera.22) The results indicated that the catalytic activity of HRP in the presence of H2O2 is useful for the derivatization of a nonfluorescent substrate into a fluorescent compound.

Accordingly, in the present study, we selected nonfluorescent QA as a substrate of HRP and investigated whether QA could be converted into a fluorescent compound by the catalytic activity of HRP in the presence of excess H2O2, and whether QA could be determined by measuring the fluorescence intensity of the reaction product. No fluorometric
method for QA using the catalytic activity of HRP in the presence of excess \( \text{H}_2\text{O}_2 \) was demonstrated previously.

**MATERIALS AND METHODS**

**Reagents** — QA was purchased from Seikagaku Kogyo Co. (Tokyo, Japan) and used without further purification. A QA solution was prepared by diluting a 1.0 mM stock solution of QA to the desired concentration with distilled water. HRP (EC 1.11.1.7, type VI, Reinheitszahl: ca. 3.0) was purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). HRP solution was prepared by diluting 100 U/ml of HRP stock solution to the desired concentration with distilled water. Control serum I Wako (BR) and control serum II Wako (BR) were purchased from Wako Pure Chemical Industries Co. (Osaka, Japan). All other chemicals were of analytical or reagent grade and used without further purification.

The buffer solutions used were (1/25 M \( \text{H}_3\text{PO}_4 \), 1/25 M \( \text{CH}_3\text{COOH} \), 1/25 M \( \text{H}_3\text{BO}_3 \))-0.2 M NaOH for pH 3.0–11.0, 0.1 M lactic acid–0.1 M sodium lactate for pH 4.0–5.3, 0.1 M \( \text{CH}_3\text{COOH} \)-0.1 M \( \text{CH}_3\text{COONa} \) for pH 4.0–6.0, and 1/15 M \( \text{KH}_2\text{PO}_4 \)-1/15 M Na\(_2\text{HPO}_4 \) for pH 5.3–6.0.

**Instruments** — The fluorescence spectra and intensities were recorded on a Shimadzu RF-5300 spectrofluorometer (Kyoto, Japan) with a quartz cell (1 cm \( \times \) 1 cm cross-section) equipped with a xenon lamp and dual monochromator. For all the experiments in this study, the bandwidths for both excitation and emission were set at 15 nm, unless otherwise noted.

**HRP Method for Determining QA** — HRP solution (1.0 ml, 10 U/ml) was added to a mixture containing sample solution (1.0 ml, 1.0–5.0 nmol of QA), 0.5 M \( \text{H}_2\text{O}_2 \) solution (1.0 ml) and 0.1 M lactate buffer solution (3.0 ml, pH 5.0). The mixture was incubated at 30°C for 90 min without exposure to light. The fluorescence intensity of the solution was measured with excitation and emission wavelengths at 328 and 377 nm, respectively. The fluorescence intensity of a reagent blank solution was similarly measured under the same conditions.

**Removal of Serum Protein from Control Serum I and II** — A mixture of serum (1.0 ml) and 0.3 M \( \text{HClO}_4 \) (9.0 ml) was thoroughly stirred and then allowed to stand for 10 min in ice. After the solution was centrifuged at 3000 rpm for 10 min, the supernatant (5.0 ml) of the reaction mixture was neutralized with 2 M NaOH, and its total volume was adjusted to 10.0 ml with 0.1 M lactate buffer (pH 5.0). One milliliter of this solution was used as a sample solution for the HRP method for determining QA.

**RESULTS AND DISCUSSION**

**Investigation of Optimum Conditions for Fluorescent Derivatization of QA with HRP**

Mawatari et al. investigated the photochemical conversion reaction of tryptophan metabolites into a fluorescent compound under various conditions and demonstrated that QA was converted into a fluorescent compound by a photochemical reaction through UV irradiation in the presence of \( \text{H}_2\text{O}_2 \). Accordingly, all reaction solutions containing QA, HRP, and \( \text{H}_2\text{O}_2 \) were incubated without exposure to light to avoid this photochemical reaction of QA in the presence of \( \text{H}_2\text{O}_2 \).

To investigate whether QA could be converted into a fluorescent compound by the catalytic activity of HRP in the presence of \( \text{H}_2\text{O}_2 \), the fluorescence intensities of reaction solutions containing QA and HRP were measured under various conditions. All solutions without HRP and/or \( \text{H}_2\text{O}_2 \) showed no or almost no fluorescence under the conditions indicated in the HRP method. Figure 1 shows the excitation and emission spectra of reaction solution containing QA, HRP, and \( \text{H}_2\text{O}_2 \) after incubation un-
under the same conditions. These results indicate that QA was converted into a fluorescent compound with maximum excitation and emission wavelengths at 328 nm and 377 nm, respectively. Accordingly, it is apparent that QA was converted into a fluorescent compound by the catalytic activity of HRP in the presence of H$_2$O$_2$ with no exposure to light, which can be determined by measuring the fluorescence intensity of the reaction product. The fluorescence intensity of the reaction product decreased gradually on standing without exposure to light. As almost the same fluorescence intensity was observed within 30 min on standing without exposure to light, the fluorescence intensity of the reaction solutions was immediately observed after incubation.

As described previously, HRP catalyzes reaction (1), in which the substrate acts as a proton donor to be oxidized into the reaction product with H$_2$O$_2$. In methods for the fluorometric determination of H$_2$O$_2$ using reaction (1), it is well known that the substrates, such as 3-$(p$)-hydroxyphenyl)propionic acid and homovanillic acid, act as mono-proton donors in the reaction and are oxidized to produce a fluorescent dimer.\(^{25,26}\) Analogous with these substrates, QA may participate as a mono-proton donor in reaction (1) and be oxidized with H$_2$O$_2$ to produce a fluorescent dimer. As described above, Mawatari et al.\(^{17}\) showed that QA was converted into a fluorescent compound (excitation: 326 nm, emission: 380 nm), with a structure not reported in a photochemical reaction through UV irradiation in the presence of H$_2$O$_2$.\(^{17}\) It is not clear whether the fluorescent compound produced in this study is exactly the same as that produced previously,\(^{17}\) although both the excitation and emission wavelengths were almost the same.

To establish the optimum conditions for the fluorescent derivatization of QA with HRP in the presence of H$_2$O$_2$, experiments were carried out using the HRP method for determining QA with 5.0 nmol of QA.

**Effects of pH and Buffers**

As the present reaction is an enzymatic reaction catalyzed by HRP, the pH and buffers of the reaction mixture may affect the fluorescent derivatization reaction. The effects of the pH and buffers on the conversion of QA into the fluorescent compound were investigated in different buffer solutions between pH 3.0 and 11.0. Figure 2 shows the fluorescence intensities of the solutions obtained in each buffer solution. The highest intensity was observed in lactate buffer solution of pH 5.0. Based on these results, a pH 5.0 lactate buffer solution was selected as the optimum pH and buffer for the fluorescent derivatization of QA.

**Effects of Concentration of H$_2$O$_2$ and HRP**

HRP is well known to exhibit its conventional catalytic activity based on the reaction cycle (native form $\rightarrow$ compound I $\rightarrow$ compound II $\rightarrow$ native form) in the presence of small amounts of the H$_2$O$_2$ to HRP ratio. In this reaction system, active compounds I and II as transient intermediates play an important role in the oxidation of various substrates. However, in the presence of excess H$_2$O$_2$ relative to HRP, it is well known that other forms, such as compound III and P-670, are formed in addition to compounds I and II.\(^{27-30}\) These results indicate that the concentration of H$_2$O$_2$ in reaction solutions affect the fluorescent derivatization of QA with HRP.

First, experiments were carried out in the presence of small amounts of H$_2$O$_2$ relative to HRP. However, no fluorescent derivatization of QA was observed even after incubation at 30°C for 120 min. As shown in Fig. 3, the fluorescent derivatization of QA was observed in the presence of an excess H$_2$O$_2$ to HRP ratio. Although an increase in fluorescence intensities was observed with increasing concentrations of H$_2$O$_2$, the fluorescence intensity reached its highest value and remained constant at H$_2$O$_2$ con-
centrations greater than 0.45 M. Thus as the concentration of H$_2$O$_2$, 0.5 M was selected.

The UV spectra of HRP in reaction solutions were measured. The characteristic bands for the native form of HRP were observed at 404, 500, and 639 nm (data not shown). With the addition of excess H$_2$O$_2$ to this HRP solution, the spectrum of HRP changed, and only the characteristic absorption bands for compound III were observed at 414, 545, and 578 nm. After the addition of QA to this solution and incubation at 30°C, the spectrum of HRP changed further, and the characteristic absorption band for P-670 in addition to compound III was observed at 670 nm. In general, compound III and P-670 are regarded as inactive forms of HRP. However, Tamura and Yamazaki demonstrated that compound III was less active than compound II, but could oxidize substrates, such as indoleacetic acid and dimethyl-p-phenylenediamine.31) Accordingly, in this study, it may be possible that compound III participated in the fluorescent derivatization of QA with HRP in the presence of excess H$_2$O$_2$.

As shown in Fig. 4, the fluorescent derivatization of QA was affected by the concentration of HRP. As the fluorescence intensities reached the highest values and remained constant at greater than 8 U/ml HRP, a 10 U/ml HRP solution was selected.

**Effects of Incubation Time and Temperature**

The effects of the incubation time for the fluorescent derivatization of QA were investigated in the range of 30–180 min. The fluorescence intensities gradually increased with increasing incubation time, and became almost constant and maximum at incubation time of greater than 90 min. Thus the incubation time chosen was 90 min. The present reaction proceeded very slowly in spite of an enzymatic reaction by HRP, indicating that QA has low reactivity as a substrate of HRP. The fluorescence intensities became maximal at 30°C in the range of 20–40°C of incubation temperature, and 30°C was selected as the incubation temperature.

**Fluorometric Determination of QA and Calibration Curve**

The HRP method for the fluorometric determination of QA was established based on the optimum conditions described above. Under these optimum conditions, a linear calibration curve was obtained between 1.0 and 5.0 nmol of QA in a 1.0 ml sample solution, as shown in Fig. 5. The correlation coefficient and relative standard deviation (n = 8) were 0.9955 and 3.58% for 3.0 nmol of QA in a 1.0 ml sample solution, respectively. By adjusting the bandwidths for both excitation and emission to 20 nm, the calibration curve was also linear in the range between 0.1 and 1.0 nmol of QA in a 1.0 ml sample solution. The detection limit of QA was 0.04 nmol/ml. The HRP method is therefore applicable to the fluorometric determination of trace amounts of QA. As the sensitivity of the present method was almost compatible with that of the fluo-
Fig. 5. Calibration Curves
The conditions are the same as in Fig. 1. The bandwidths for both excitation and emission: (a) 15 nm and (b) 20 nm.

Table 1. Effects of Interfering Substances on the Fluorescent Derivatization of QA with HRP in the Presence of H₂O₂ without Exposure to Light

<table>
<thead>
<tr>
<th>Substance</th>
<th>Added (nmol)</th>
<th>Error (%)&lt;sup&gt;a&lt;/sup&gt;</th>
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<th>Added (nmol)</th>
<th>Error (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>34.4 I.U.</td>
<td>+5.4</td>
<td>NH₂⁺</td>
<td>100</td>
<td>−4.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>100</td>
<td>−3.6</td>
<td>PO₄³⁻</td>
<td>100</td>
<td>+1.8</td>
</tr>
<tr>
<td>Albumin (HSA)</td>
<td>20 mg</td>
<td>−86.2</td>
<td>K⁺</td>
<td>100</td>
<td>−3.8</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>100</td>
<td>−39.6</td>
<td>Ca²⁺</td>
<td>100</td>
<td>+2.5</td>
</tr>
<tr>
<td>Citric acid</td>
<td>100</td>
<td>+0.2</td>
<td>Fe³⁺</td>
<td>100</td>
<td>−4.3</td>
</tr>
<tr>
<td>EDTA</td>
<td>100</td>
<td>−101.8</td>
<td>CO₂⁻</td>
<td>100</td>
<td>−1.3</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>100</td>
<td>+0.7</td>
<td>Br⁻</td>
<td>100</td>
<td>−2.0</td>
</tr>
<tr>
<td>3-Indoleacetic acid</td>
<td>100</td>
<td>−4.7</td>
<td>I⁻</td>
<td>100</td>
<td>−92.4</td>
</tr>
<tr>
<td>Kynurenic acid</td>
<td>100</td>
<td>—&lt;sup&gt;b&lt;/sup&gt;</td>
<td>F⁻</td>
<td>100</td>
<td>0.0</td>
</tr>
</tbody>
</table>

QA added, 5.0 nmol. <sup>a</sup>Error (%) = 100 × (QA(found) − QA(added))/QA(added). <sup>b</sup>A fluorescence spectrum derived from a fluorescent compound (excitation: 367 nm, emission: 470 nm) produced by the oxidation of kynurenic acid with HRP was observed.

orometric method of Mawatari et al.,<sup>17</sup> it will be satisfactory for the determination of trace amounts of QA in vital fluids. According to the literature, the amounts of QA in urine and blood samples of a healthy individual are 36–78 µmol/day<sup>27</sup> and 0.47 ± 0.047 µmol/l, respectively.<sup>12</sup> Although pretreatment, such as the concentration of vital fluid samples or extraction of QA from vital fluid samples, is required, the HRP method may be applied to the determination of QA in human vital fluid samples.

**Effects of Interfering Substances**

Interference in the HRP method was investigated using various substances. As HRP is originally an enzyme associated with oxidation-reduction reaction systems, some reducing and oxidizing substances may affect the fluorescent derivatization of QA with HRP in the presence of H₂O₂. As shown in Table 1, reducing substances such as ascorbate and iodide ions caused severe interference. Moreover, human serum albumin (HSA) caused severe interference. For the determination of trace amounts of QA in a vital sample, the removal of protein from vital samples is thus necessary. In the presence of L-tryptophan and its metabolites, L-tryptophan and 3-indoleacetic acid showed no interference. However, kynurenic acid showed severe interference because kynurenic acid was converted into a fluorescent compound with HRP under the conditions in this study, as described in the previous report.<sup>22</sup> Other substances and ions except for EDTA showed almost no interference.
Application to the Determination of QA in Control Sera

To confirm whether the HRP method is applicable to actual samples of vital fluids, the determination of QA spiked in control serum I and II was investigated using the HRP method. Since the HRP method was affected by HSA, as shown in Table 1, serum proteins were removed from solutions in control sera before the determination of QA. QA was added to control serum I and II prior to the removal of serum proteins, and each solution was used as a sample solution in the HRP method. The recovery rates of QA (6.0 nmol/ml) added to each serum were 99.7 ± 0.8% (n = 3) and 98.0 ± 0.7% (n = 3) for control serum I and II, respectively. The results were satisfactory for both.

In conclusion, QA was converted to the fluorescent compound by the catalytic activity of HRP in the presence of H$_2$O$_2$ without exposure to light. The HRP method using this fluorescent derivatization of QA was useful for the determination of trace amounts of QA. To the best of our knowledge, this is the first example of fluorometric determination of QA containing various substances and ions.

REFERENCES


