Spectrofluorometric Determination of Kynurenic Acid with Horseradish Peroxidase in the Presence of Hydrogen Peroxide

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The catalytic activity of horseradish peroxidase (HRP) in the presence of hydrogen peroxide has been investigated for the fluorescent derivatization of kynurenic acid under conditions with no exposure to light. Non-fluorescent kynurenic acid was converted into a fluorescent compound (Ex: 367 nm, Em: 470 nm) with HRP in the presence of hydrogen peroxide, and the optimum conditions of this fluorescent derivatization were investigated. Moreover, this fluorescent derivatization was developed for a spectrofluorometric determination of trace amounts of kynurenic acid by measuring the fluorescence intensity of the fluorescent compound. The calibration curve obtained was linear from 1.0 to 10.0 nmol of kynurenic acid in a 1.0 mL sample solution. The relative standard deviation at 5.0 nmol of kynurenic acid was 5.71% ($n = 5$). By adjusting the bandwidths for both the excitation and emission to 15 nm, the calibration curve was also linear in the range between 0.1 to 1.0 nmol of kynurenic acid in a 1.0 mL sample solution. This method was applied to the fluorometric determination of trace amounts of kynurenic acid in the control sera.

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Introduction

Horseradish peroxidase (HRP) is well known to catalyze the following reaction (1), in which substrates (AH_2) are oxidized to a corresponding reaction product (A) by the catalytic activity of HRP in the presence of H_2O_2 . In general, reaction (1) is routinely applied to the photometric or fluorometric determination of trace amounts of H_2O_2 in various fields of chemistry, such as clinical, environmental and food chemistry.¹⁻⁴

For the fluorometric determination of trace amounts of H_2O_2 , some non-fluorescent fluorochromogen such as 3-(*p*-hydroxyphenyl)propionic acid and homovanillic acid as AH_2 were selected, and the fluorescence intensity of the corresponding fluorescent compounds produced through reaction (1) was measured.^{5,6} In these cases, reaction (1) proceeded quantitatively in the presence of excess AH_2 to trace amounts of H_2O_2 . Conversely, if reaction (1) will proceed quantitatively, even in the presence of excess H_2O_2 to trace amounts of AH_2 , AH_2 will be determinable by measuring the reaction product (A). Accordingly, for developing a fluorometric determination of trace amounts of non-fluorescent AH_2 , we tried to apply reaction (1), in which a non-fluorescent substrate could be converted into a fluorescent product by the catalytic activity of HRP in the presence of excess H_2O_2 .

Kynurenic acid (KA) is non-fluorescent, one of the metabolites of tryptophan, and is of great interest in terms of its connection with various diseases, such as diabetes and vitamin

B_6 deficiency.⁷⁻¹⁰ Therefore, it is very important to measure the concentration of KA in vital fluids for determining the proper diagnosis of such diseases. So far, for measuring trace amounts of KA in vital fluids, various methods have been demonstrated regarding photometric and fluorometric determinations, and so on.¹¹⁻¹⁷ In particular, Watanabe *et al.* have demonstrated fluorometric methods that involve an enhancement of the fluorescence intensity of KA by adding a zinc acetate reagent, or using the conversion of KA into a fluorescent compound by ultraviolet irradiation in the presence of H_2O_2 .^{14,15} These methods have been applied to the determination of KA in vital fluids, such as plasma, urine and cerebrospinal fluid. However, some disadvantages still remain regarding the irradiation time of ultraviolet light and a waste containing zinc ion.

Accordingly, in an attempt to develop a new method for fluorometric determination of trace amounts of KA, we selected non-fluorescent KA as the substrate, and investigated whether KA will be converted into a fluorescent compound during reaction (1) and moreover KA can be determined by measuring the fluorescence intensity of the reaction product. To the best of our knowledge, no fluorometric method using the catalytic activity of HRP in the presence of H_2O_2 has been demonstrated for the determination of trace amounts of KA.

Experimental

Reagents and apparatus

KA was purchased from Seikagaku Kogyo Co. and used without further purification. HRP, and control serum I and II were purchased from Wako Pure Chemical Industries Co. All other chemicals were of analytical or reagent grade, and used without further purification.

The fluorescence spectra and fluorescence intensities were recorded on a Shimadzu RF-5300 spectrofluorometer with a

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Table 1 Fluorescent derivatization of KA under various conditions

Sample solution (total: 5.0 mL)	KA/ nmol	HRP/ units	H ₂ O ₂ / μmol	Fluorescence intensity ^a /a.u.
Solution (A)	5.0	2.0	50	400
Solution (B)	5.0	2.0	0	~0
Solution (C)	5.0	0	50	~0
Solution (D)	5.0	0	0	~0

a. The intensity was measured at excitation and emission wavelengths of 367 and 470 nm, respectively. All solutions were incubated for 30 min in a pH 6.0 phosphate buffer at room temperature without exposure to light.

quartz cell (1 cm × 1 cm cross-section) equipped with a xenon lamp and a dual monochromator. For all experiments in this study, the bandwidths for both excitation and emission were set at 10 nm, unless otherwise noted.

HRP method for determination of KA

A HRP solution (1.0 mL, 2.0 U mL⁻¹) was added to a mixture containing a sample solution (1.0 mL, 1.0–10.0 nmol of KA), a 50 mM H₂O₂ solution (1.0 mL) and a 1/15 M phosphate buffer solution (3.0 mL, pH 6.0); the mixture was incubated at 35°C for 30 min without exposure to light. The fluorescence intensity of the mixture solution was measured at excitation and emission wavelengths of 367 and 470 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 0.6 M HClO₄ (4.0 mL) was adequately stirred, and then allowed to stand for 10 min in ice. After the solution was centrifuged at 3000 rpm for 10 min, the supernatant (2.5 mL) of the reaction mixture was neutralized with 2 N NaOH, and its total volume was adjusted to 5.0 mL with a 1/15 M phosphate buffer (pH 6.0). One milliliter of this solution was used as a sample solution for the HRP method.

The buffer solutions used were 1/15 M KH₂PO₄–1/15 M Na₂HPO₄ for pH 5.0–8.0. Buffer solutions used for pH 6.0 were (1/25 M H₃PO₄, 1/25 M CH₃COOH, 1/25 M H₃BO₃)–0.2 M NaOH, 0.1 M NH₄Cl–0.1 M NH₄OH, (0.2 M H₃BO₃, 0.05 M NaCl)–0.05 M Na₂B₄O₇, and 0.1 M HCl–0.05 M Na₂B₄O₇.

Results and Discussion

Fluorescent derivatization of KA with HRP

Mawatari *et al.* have demonstrated that KA, a non-fluorescent compound, was converted into a fluorescent compound by a photochemical reaction through ultraviolet irradiation in the presence of H₂O₂.¹⁵ This indicates that KA is very sensitive to the irradiation of ultraviolet light, and is easily converted into a fluorescent compound by a photochemical reaction. Accordingly, in this study, all experiments were carried out under the condition of no exposure to light in order to avoid this photochemical reaction of KA with H₂O₂.

As shown in Table 1, the fluorescence intensities of reaction solutions containing KA under various conditions were measured for investigating whether KA will be converted into a fluorescent compound with HRP in the presence of H₂O₂ or not. All solutions were incubated for 30 min in a pH 6.0 phosphate buffer at room temperature, and their fluorescence intensities

Fig. 1 Excitation and emission spectra of a fluorescent compound produced with HRP in the presence of H₂O₂ without exposure to light. After a mixture containing a KA solution (1.0 mL, 5.0 μmol), a 50 mM H₂O₂ solution (1.0 mL), 2.0 U mL⁻¹ HRP solution (1.0 mL) and 1/15 M phosphate buffer solution (3.0 mL, pH 6.0) was incubated at 35°C for 30 min without exposure to light, the fluorescence intensity of the mixture was measured. The spectra (A and B) show the respective blank fluorescence.

were measured at excitation and emission wavelengths of 367 and 470 nm, respectively. As shown in Table 1, solution (D) containing only KA showed no fluorescence. Only solution (A) containing both HRP and H₂O₂ showed strong fluorescence, whereas solutions (B) and (C) without HRP or H₂O₂ showed almost no fluorescence. It is apparent that KA was converted into the fluorescent compound only in the presence of both HRP and H₂O₂, even without exposure to light, and is capable of being determined fluorometrically by measuring the fluorescence intensity of the reaction product during reaction (1).

As described previously, Mawatari *et al.*¹⁵ has shown that KA was converted into a fluorescent compound by a photochemical reaction through ultraviolet irradiation in the presence of H₂O₂. The excitation and emission wavelengths of this fluorescent compound are 370 and 465 nm, and are almost the same as that of a fluorescent product in this study, respectively. However, it is not clear whether the fluorescent compound described in the literature¹⁵ is quite same as that produced in this study or not, because no mention is made of its structure of the fluorescent compound in the literature.¹⁵ In reaction (1), where HRP catalyzes, it is well known that the substrate acts as a proton donor to be oxidized into the reaction product with H₂O₂. Generally, substrates are known to act not only as a two-proton donor (AH₂) but also as a mono-proton donor (AH) in reaction (1). For example, non-fluorescent 3-(*p*-hydroxyphenyl)-propionic acid and homovanillic acid as substrates have been shown to act as a mono-proton donor to be oxidized into the corresponding fluorescent dimer, according to reaction (1).^{18,19} In analogy with 3-(*p*-hydroxyphenyl)propionic acid and homovanillic acid, KA may be oxidized with H₂O₂ and condensed to produce a fluorescent dimer, although the structure of the reaction product in this study is still not clear. Because HRP is not always so specific in its choice of substrates, KA may participate as a mono-proton donor in reaction (1) to be oxidized into a fluorescent dimer.

Investigation of optimum conditions for the fluorescent derivatization of KA with HRP

In order to establish the optimum conditions for the

Table 2 Effects of the buffer on the fluorescent derivatization of KA with HRP at pH 6.0 in the presence of H_2O_2 without exposure to light

Buffer (pH 6.0)	Fluorescence intensity/a.u.
1/15 M KH_2PO_4 -1/15 M Na_2HPO_4	400
0.1 M NH_4Cl -0.1 M NH_4OH	295
0.05 M $\text{Na}_2\text{B}_4\text{O}_7$ -(0.2 M H_3BO_3 , 0.05 M NaCl)	250
1/25 M (H_3PO_4 , CH_3COOH , H_3BO_3)-0.2 M NaOH	110
0.05 M $\text{Na}_2\text{B}_4\text{O}_7$ -0.1 M HCl	100

fluorescent derivatization of KA with HRP in the presence of H_2O_2 , experiments by using the HRP method for determining KA with 5.0 nmol of KA were carried out without exposure to light.

Effects of the pH and buffers

The catalytic activity of HRP for the conversion of KA into a fluorescent compound was investigated in phosphate buffer solutions between pH 5 and 8. Figure 2 shows the fluorescence intensities of solutions obtained in each buffer solution. As shown in Fig. 2, HRP exhibited high levels of catalytic activity in a buffer solution of pH 6. Moreover, HRP exhibited the highest activity in a phosphate buffer solution among various buffer solutions of pH 6, as shown in Table 2. These results are reasonable because the optimum pH of HRP is about pH 6. Accordingly, a phosphate buffer solution of pH 6.0 was selected as the optimum pH and a buffer for the fluorescent derivatization of KA.

Effects of the concentration of H_2O_2 and HRP

Both concentrations of H_2O_2 and HRP affected the fluorescent derivatization of KA. The effect of the concentration of H_2O_2 for the fluorescent derivatization of KA was investigated in the range of 10 to 60 mM H_2O_2 . Although an increase of the fluorescent intensity was observed with increasing the concentration of H_2O_2 , the fluorescent intensities became almost constant, and maximum over 50 mM of H_2O_2 . The fluorescence intensities were almost constant and maximum between 2.0 and 5.0 U mL^{-1} of HRP. Thus, the concentrations of H_2O_2 and HRP were selected as 50 mM and 2.0 U mL^{-1} , respectively.

Effects of the incubation time and the incubation temperature

The effects of the incubation time and the incubation temperature for the fluorescent derivatization of KA were investigated in the range of 10 to 60 min and 25 and 45°C, respectively. The fluorescence intensities became constant and maximum after 30 min of incubation time. Thus, the incubation time was chosen to be 30 min. The fluorescence intensities became maximum at 35°C among 25 and 45°C of the incubation temperature. As the incubation temperature, 35°C was selected.

Fluorometric determination of KA and the calibration curve

Based on the optimum conditions described above for the fluorescent derivatization of KA with HRP in the presence of H_2O_2 , the HRP method for the fluorometric determination of KA was established. Under these optimum conditions, a linear calibration curve was obtained between 1.0 and 10.0 nmol of KA in a 1.0 mL sample solution, as shown in Fig. 3. The correlation coefficient and the relative standard deviation ($n = 5$) were 0.9955 and 5.71% for 5.0 nmol of KA in a 1.0 mL sample solution, respectively. By adjusting the bandwidths for both excitation and emission to 15 nm, the calibration curve was

Fig. 2 Effects of the pH on the fluorescent derivatization of KA with HRP. The conditions are same as in Fig. 1, except for the pH of a 1/15 M phosphate buffer solution.

Fig. 3 Calibration curves in the range of 1.0 to 10 μmol of KA and 0.1 to 1.0 μmol of KA in a 1.0 mL sample solution, respectively.

also linear in the range between 0.1 and 1.0 nmol of KA in a 1.0 mL sample solution. Accordingly, the HRP method is applicable to the fluorometric determination of trace amounts of KA. Although the sensitivity of the present method does not always correspond to that of the fluorometric methods employed by Watanabe *et al.*,^{14,15} it will be satisfactory for the determination of trace amounts of KA in vital fluids in conjunction with HPLC.

Effect of foreign substances

Interference in the HRP method was investigated in the presence of various foreign substances and ions. Since HRP is originally an enzyme associated with oxidation-reduction reaction systems, some reducing and oxidizing substances may affect the fluorescent derivatization of KA with HRP in the presence of H_2O_2 . Accordingly, as shown in Table 3, reducing substances, such as ascorbate and iodide ions, showed severe interference. In the case of a sample solution containing human serum albumin (HSA), the fluorescence spectrum derived from HSA was predominantly observed, because HSA is fluorescent. The removal of protein from a vital sample containing a fluorescent protein should be necessary for the determination of trace amounts of KA. Other substances and ions showed almost no interference.

Table 3 Effect of foreign substances on the fluorescent derivatization of KA with HRP in the presence of H₂O₂ without exposure to light (KA added: 1.0 nmol)

Substrate	Added/ nmol	Error, %	Substrate	Added/ nmol	Error, %
Heparin	17.3 (I.U.)	+1.8	K ⁺	10	+7.7
Glycine	10	-2.8	Ca ²⁺	10	+7.0
Albumin (HSA)	20 mg	— ^a	Fe ³⁺	10	-3.9
Ascorbic acid	10	-38.3	CO ₃ ²⁻	10	+4.1
Citric acid	10	-1.4	Br ⁻	10	-5.8
Acetic acid	10	+3.2	I ⁻	10	-44.6
NH ₄ ⁺	10	+1.0	F ⁻	10	+4.3

a. A fluorescence spectrum derived from HSA was observed.

Application to the determination of KA in sera

The HRP method was applied to the determination of KA spiked in control serum I and II. Because the HRP method was affected by HSA, as shown in Table 3, the sample solution after the removal of serum proteins in the control sera was applied to the determination of KA. KA was added to each control serum I and II prior to the removal of serum proteins, and each solution was used as a sample solution for the HRP method. The recoveries of KA added to each control serum were 92.7 ± 0.8 ($n = 3$) and 90.7 ± 0.7 ($n = 3$) for control serum I and II, respectively. The results were satisfactory for both control serum I and II. Price *et al.* have reported that the amounts of KA in urine of health adults were 13.3 ± 4.0 $\mu\text{mol day}^{-1}$ in males and 13.2 ± 1.8 $\mu\text{mol day}^{-1}$ in females.²⁰ Accordingly, the HRP method may be applied to the determination of KA in human urine sample, although the pretreatment, such as the concentration of urine sample or the extraction of KA from urine sample, should be required.

Conclusions

Non-fluorescent KA was easily converted into a fluorescent compound by the catalytic activity of HRP in the presence of H₂O₂, even without exposure to light. The HRP method using this fluorescent derivatization of KA was useful for the fluorometric determination of trace amounts of KA. This is the first example of the determination of KA using the catalytic activity of HRP in the presence of H₂O₂. Moreover, this HRP

method was also useful for the fluorometric determination of trace amounts of KA in vital fluids, such as plasma.

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