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 metabolism[1,2] 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 H_2O_2.[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 H_2O_2 was usable for the fluorescent derivatization of a nonfluorescent substrate (AH_2) into a fluorescent compound according to the following reaction (1), which is routinely applied in the determination of trace amounts of H_2O_2 in clinical and environmental analyses.[23,24]

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

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 H_2O_2 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 H_2O_2 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 H_2O_2, 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 H_2O_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 H_3PO_4, 1/25 M CH_3COOH, 1/25 M H_3BO_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 CH_3COOH–0.1 M CH_3COONa for pH 4.0–6.0, and 1/15 M KH_2PO_4–1/15 M Na_2HPO_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 × 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 H_2O_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 $HClO_4$ (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 H_2O_2.[17] Accordingly, all reaction solutions containing QA, HRP, and H_2O_2 were incubated without exposure to light to avoid this photochemical reaction of QA in the presence of H_2O_2.

To investigate whether QA could be converted into a fluorescent compound by the catalytic activity of HRP in the presence of H_2O_2, the fluorescence intensities of reaction solutions containing QA and HRP were measured under various conditions. All solutions without HRP and/or H_2O_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 H_2O_2 after incubation un-

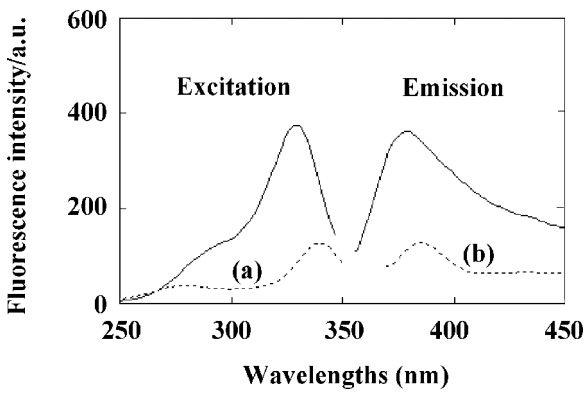

Fig. 1. Excitation and Emission Spectra of a Fluorescent Compound Produced with HRP in the Presence of H_2O_2 without Exposure to Light

After a mixture containing QA solution (1.0 ml, 5.0 nmol), 0.5 M H_2O_2 solution (1.0 ml), 10 U/ml HRP solution (1.0 ml), and 0.1 M lactate buffer solution (3.0 ml, pH 5.0) was incubated at 30°C for 90 min without exposure to light, the fluorescence intensity of the mixture was measured. Dotted lines show the fluorescence spectra of QA-free solution.

der 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_2O_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_2O_2. In methods for the fluorometric determination of H_2O_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_2O_2 to produce a fluorescent dimer. As described above, Mawatari *et al*. 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_2O_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_2O_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.

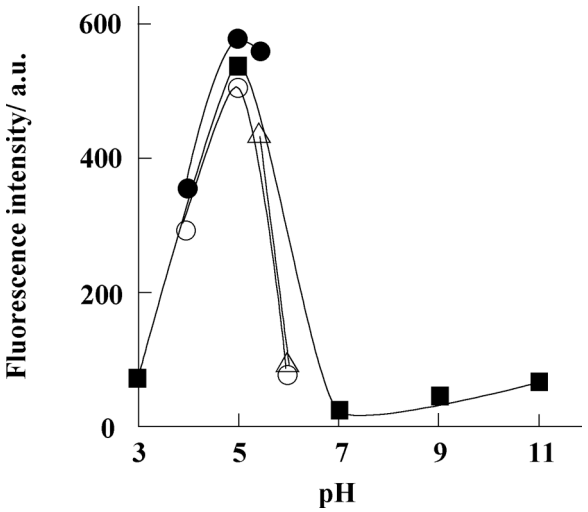

Fig. 2. Effects of pH and Buffer on the Fluorescent Derivatization of QA with HRP

The conditions are the same as in Fig. 1, except for the use of 0.1 M lactate buffer solution. ●, lactic acid–sodium lactate (pH 4.0–5.3); ■, (H_3PO_4, CH_3COOH, H_3BO_3)–NaOH (pH 3.0–11.0); ○, CH_3COOH–CH_3COONa (pH 4.0–6.0); △, KH_2PO_4–Na_2HPO_4 (pH 5.3–6.0).

Effects of Concentration of H_2O_2 and HRP

HRP is well known to exhibit its conventional catalytic activity based on the reaction cycle (native form → compound I → compound II → native form) in the presence of small amounts of the H_2O_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_2O_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_2O_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_2O_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_2O_2 to HRP ratio. Although an increase in fluorescence intensities was observed with increasing concentrations of H_2O_2, the fluorescence intensity reached its highest value and remained constant at H_2O_2 con-

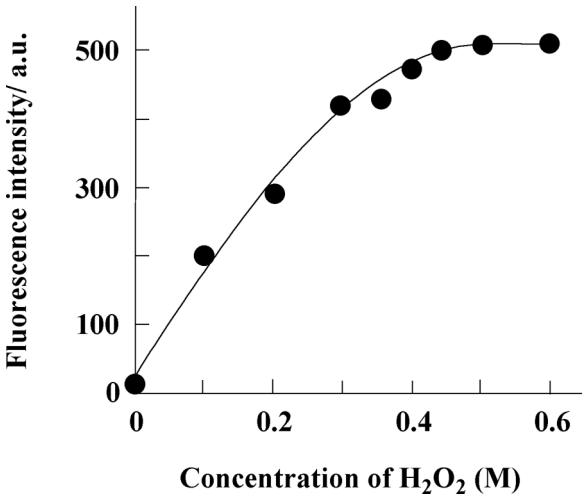

Fig. 3. Effects of the Concentration of H_2O_2 on the Fluorescent Derivatization of QA with HRP in the Presence of H_2O_2

The conditions are the same as in Fig. 1, except for the use of 0.5 M H_2O_2 solution.

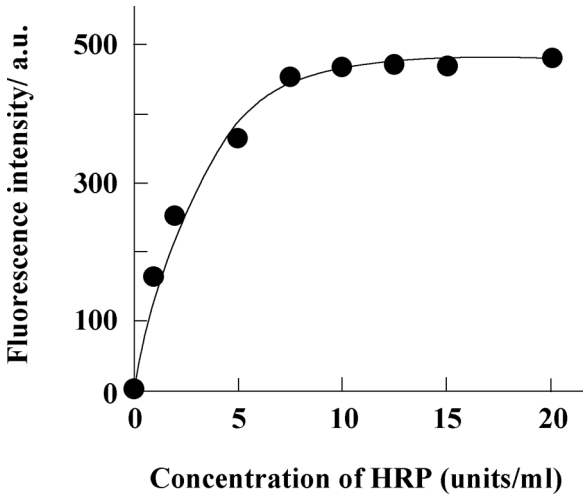

Fig. 4. Effects of the Concentration of HRP on the Fluorescent Derivatization of QA with HRP in the Presence of H_2O_2

The conditions are the same as in Fig. 1, except for the use of 10 U/ml HRP solution.

centrations greater than 0.45 M. Thus as the concentration of H_2O_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_2O_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_2O_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 flu-

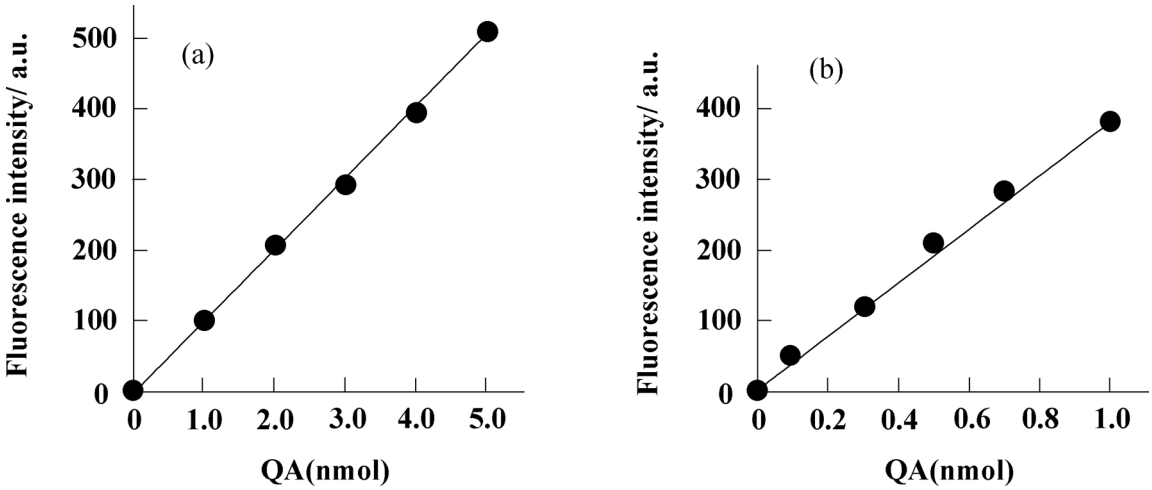

Fig. 5. Calibration Curves
The conditions are the same as in Fig. 1. The bandwidths for both excitation and emission: (a) 15 nm and (b) 20 nm.

Table 1. Effects of Interfering Substances on the Fluorescent Derivatization of QA with HRP in the Presence of H_2O_2 without Exposure to Light

Substance	Added (nmol)	Error (%)[a)]	Substance	Added (nmol)	Error (%)[a)]
Heparin	34.4 International unit (I.U.)	+5.4	NH_4^+	100	−4.8
Glycine	100	−3.6	PO_4^{3-}	100	+1.8
Albumin (HSA)	20 mg	−86.2	K^+	100	−3.8
Ascorbic acid	100	−39.6	Ca^{2+}	100	+2.5
Citric acid	100	+0.2	Fe^{3+}	100	−4.3
EDTA	100	−101.8	CO_3^{2-}	100	−1.3
L-Tryptophan	100	+0.7	Br^-	100	−2.0
3-Indoleacetic acid	100	−4.7	I^-	100	−92.4
Kynurenic acid	100	—[b)]	F^-	100	0.0

QA added, 5.0 nmol. *a*) Error (%) = 100 × {QA(found)−QA(added)}/QA(added). *b*) 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.*,[17] 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[27] and 0.47 ± 0.047 μmol/l, respectively.[12] 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 H_2O_2. 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.[22] 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 $99.7 \pm 0.8\%$ ($n = 3$) and $98.0 \pm 0.7\%$ ($n = 3$) 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_2O_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 using the catalytic activity of HRP in the presence of H_2O_2. Moreover, the HRP method was also useful for the fluorometric determination of trace amounts of QA even in vital fluid such as plasma containing various substances and ions.

REFERENCES

1) Nyhan, W. L. and Haas, R. (1997) Inborn errors of amino acid metabolism and transport. In *Molecular and Genetic Basis of Neurological Disease* (Rosenberg, R. N., Ed.), Butterworth-Heinemann, Boston, pp.1129–1150.
2) Kim, W., Erlandsen, H., Surendran, S., Stevens, R. C., Gamez, A., Michols-Matalon, K., Tyring, S. K. and Matalon, R. (2004) Trends in enzyme therapy for phenylketouria. *Mol. Ther.*, **10**, 220–224.
3) Kato, A., Suzuki, K. and Sato, S. (1992) Imbalance of amino acid metabolism in fulminant hepatitis and its management. *Japanese Journal of Clinical Medicine*, **50**, 1599–1603 (in Japanese).
4) Castellino, P., Solini, A., Luzi, L., Barr, J. G., Smith, D. J., Petrides, A., Giordano, M., Carroll, C. and DeFronzo, R. A. (1992) Glucose and amino acid metabolism in chronic renal failure: effect of insulin and amino acids. *Am. J. Physiol.*, **262**, F168–F176.
5) Dejong, C. H. C., van de Poll, M. C. G., Soeters, P. B., Jalan, R. and Damink, S. W. M. O. (2007) Aromatic amino acid metabolism during liver failure. *J. Nutr.*, **137**, 1579S–1585S.
6) Medana, I. M., Day, N. P., Salahifar-Sabet, H., Stocker, R., Smythe, G., Bwanaisa, L., Njobvu, A., Kayira, K., Turner, G. D. and Taylor, T. E. (2003) Metabolites of the kynurenine pathway of tryptophan metabolism in the cerebrospinal fluid of Malawian children with malaria. *J. Infect. Dis.*, **188**, 844–849.
7) Ohashi, H., Saito, K., Fujii, H., Wada, H., Furuta, N., Takemura, M., Maeda, S. and Seishima, M. (2004) Changes in quinolinic acid production and its related enzymes following D-galactosamine and lipopolysaccharide-induced hepatic injury. *Arch. Biochem. Biophys.*, **428**, 154–159.
8) Guilemin, G. J., Brew, B. J., Noonan, C. E., Knight, T. G., Smythe, G. A. and Cullen, K. M. (2007) Mass spectrometric detection of quinolinic acid in microdissected Alzheimer's disease plaques. *Int. Congr. Ser.*, **1304**, 404–408.
9) Guidetti, P., Bates, G. P., Graham, R. K., Hayden, M. R., Leavitt, B. R., MacDonald, M. E., Slow, E. J., Wheeler, V. C., Woodman, B. and Schwarcz, R. (2006) Elevated brain 3-hydroxykynurenine and quinolinate levels in Huntington disease mice. *Neurobiol. Dis.*, **23**, 190–197.
10) Guillemin, G. J., Meininger, V. and Brew, B. J. (2005) Implications for the kynurenine pathway and quinolinic acid in amyotrophic lateral sclerosis. *Neurodegenerative Diseases*, **2**, 166–176.
11) Brew, B. J., Corbeil, J., Pemberton, L., Heyes, M., Evans, L., Penny, R. and Cooper, D. A. (1992) Quinolinic acid and the pathogenesis of AIDS dementia. *International Conference on AIDS*, **8**, 19–24.
12) Smythe, G. A., Poljak, A., Bustamante, S., Braga, O., Maxwell, A., Grant, R. and Sachdev, P. (2003) ECNI GC-MS analysis of picolinic and quinolinic acids and their amides in human plasma, CFS, and brain tissue. *Adv. Exp. Med. Biol.*, **527**, 705–712.
13) Toseland, P. A. (1969) The determination of urinary quinolinic acid by gas-liquid chromatography. *Clin. Chim. Acta*, **25**, 185–186.
14) Dobbie, M., Crawley, J., Waruiru, C., Marsh, K. and Surtees, R. (2000) Cerebrospinal fluid studies in children with cerebral malaria: An excitotoxic mechanism?. *Am. J. Trop. Med. Hyg.*, **62**, 284–290.
15) McDaniel, H. G., Huey, W. J., Boshell, B. R. and Buris, R. (1972) Improved method for measuring quinolinic acid in biological specimens. *Anal. Biochem.*, **49**, 373–378.
16) Watanabe, M. (2002) Microanalysis of tryptophan metabolites and suppressor factor of delayed-type

hypersensitivity in mice. *Yakugaku Zasshi*, **122**, 429–434 (in Japanese).
17) Mawatari, K., Oshida, K., Iinuma, F. and Watanabe, M. (1995) Determination of quinolinic acid in human urine by liquid chromatography with fluorometric detection. *Anal. Chim. Acta*, **302**, 179–183.
18) Taguchi, H., Koyama, S., Shimabayashi, Y. and Iwai, K. (1983) A new fluorometric assay method for quinolinic acid. *Anal. Biochem.*, **131**, 194–197.
19) Presits, P. and Molnar-Perl, I. (2003) HPLC of tryptophan and its metabolites using simultaneously UV, native fluorescence and pre-column fluorescence derivatization. *Chromatographia*, **57**, S/87–S/92.
20) Xia, C., Dang, Y. and Brown, O. R. (1998) HPLC analysis of quinolinic acid, a NAD biosynthesis intermediate, after fluorescence derivatization in an aqueous matrix. *Microbios*, **94**, 167–181.
21) Wolfensberger, M., Amsler, U., Cuenod, M., Foster, A. C., Whetsell, W. O., Jr. and Schwarcz, R. (1983) Identification of quinolinic acid in rat and human brain tissue. *Neurosci. Lett.*, **41**, 247–252.
22) Odo, J., Funai, T. and Hirai, A. (2007) Spectrofluorometric determination of kynurenic acid with horseradish peroxidase in the presence of hydrogen peroxide. *Anal. Sci.*, **23**, 317–320.
23) Kanai, M., Okabe, H., Sekiguchi, M., Nomoto, S., Kameko, M., Isobe, M., Tozuka, M., Hidaka, H., Oguchi, M. and Kawa, S. (1998) Clinical chemistry examination. In *Rinsyo-Kensaho-Teiyo* (Kanai, I. and Kanai, M., Eds.), Kinbara Press, Tokyo, chap. 6, pp.459–682 (in Japanese).
24) Navas, M. J., Jimenez, A. M. and Galan, G. (1999) Air analysis: determination of hydrogen peroxide by chemiluminescence. *Atmos. Environ.*, **33**, 2279–2283.
25) Guilbault, G. G., Brignac, P. J. and Juneau, M. (1968) New substrates for the fluorometric determination of oxidative enzymes. *Anal. Chem.*, **40**, 1256–1263.
26) Kusu, F., Tsuneta, T. and Takamura, K. (1990) Fluorometric determination of serum cholinesterase activity. *Bunseki Kagaku*, **39**, 115–121 (in Japanese).
27) Kawano, T., Muto, S., Adachi, M., Hosoya, H. and Lapeyrie, F. (2002) Spectroscopic evidence that salicylic acid converts a temporally inactivated form of horseradish peroxidase (compound III) to the irreversibly inactivated verdohemoprotein (P-670). *Biosci. Biotechnol. Biochem.*, **66**, 646–650.
28) Arnao, M. B., Acosta, M., del Rio, J. A., Varon, R. and Garcia-Canovas, F. (1990) A kinetic study on the suicide inactivation of peroxidase by hydrogen peroxide *Biochim. Biophys. Acta*, **1041**, 43–47.
29) Adediran, S. A. (1996) Kinetics of the formation of p-670 and of the decay of compound III of horseradish peroxidase. *Arch. Biochem. Biophys.,* **327**, 279–284.
30) Goodwin, D. C., Grover, T. A. and Aust, S. D. (1997) Roles of efficient substrates in enhancement of peroxidase-catalyzed oxidations. *Biochemistry*, **36**, 139–147.
31) Tamura, M. and Yamazaki, I. (1972) Reactions of the oxyform of horseradish peroxidase. *J. Biochem.* (Tokyo), **71**, 311–319.