Development of a Novel Fluorescence Reaction for Specific Determination of Orotic Acid in a Bio-sample and a Fluorometric Assay of Dihydroorotate Dehydrogenase

A thesis submitted to the Graduate School of Biomedical Science, Nagasaki University, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Pharmaceutical Sciences

By

Sheng Yin

Laboratory of Chemistry of Biofunctional Molecules
Department of Pharmaceutical Health Sciences
Graduate School of Biomedical Sciences
Nagasaki University, Japan

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Abbreviations

BAO	Benzamidoxime
CPS	Carbamoyl phosphate synthetase
DCPIP	2,6-Dichloroindophenol
DHO	Dihydroorotic acid
DHODH	Dihydroorotate dehydrogenase
FL	Fluorescence
HPLC	High-performance liquid chromatography
OCT	Ornithine transcarbamoylase
4-TFMBAO	4-(Trifluoromethyl)benzamidoxime
UMP	Uridine monophosphate
UMPS	Uridine monophosphate syntetase

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Abstract

Orotic acid is recognized as a marker for diagnosis of orotic aciduria, an autosomal recessive disorder. At the same time, it is also an intermediate in the de novo biosynthetic pathway of pyrimidine. Thus, lots of detection methods, including columns switching HPLC coupling with UV detector, GS-MS, chemical derivatization, colorimetric methods, have been developed for the determination of orotic acid. However, these methods either take long time or require expensive equipment. Besides, some of these methods are of low selectivity or sensitivity for determinations of orotic acid. For diagnosis of orotic aciduria, a high-throughput method that is facile to conduct is still required.

As the enzyme catalyzes the rate-limiting step of the de novo biosynthetic pathway of pyrimidine, dihydroorotate dehydrogenase (DHODH) has been studied over the past decades. These studies have revealed that the enzyme plays a significant role for the proliferation of parasite and virus in an infected host, as well as growth of tumor, since inhibition of this enzyme have shown anti-malaria, anti-virus and anti-tumor effects. So far, a colorimetric assay for the enzyme is widely used for the screening of effective synthetized inhibitors. However, the method is limited that recombination protein after purification was used since the dye used for the colorimetric assay was easily influenced by cellular reducing components.

In the first part of the present study, I developed a spectrofluorometric method with or without high performance liquid chromatography for the selective and sensitive quantification of orotic acid in human biological specimens, using 4-trifluoromethylbenzamidoxime (4-TFMBAO) as a fluorogenic reagent. This reagent provided intensive fluorescence for only orotic acid amongst 62 compounds including structurally related bio-substances such as nucleic acid bases, nucleosides, nucleotides, amino acids, vitamins, bilirubin, uric acid, urea, creatine, creatinine and sugars. Under optimized reaction conditions, orotic acid was reacted with 4-TFMBAO, $K_3[Fe(CN)_6]$ and K_2CO_3 in an aqueous solution. The fluorescence produced from the orotic acid derivative was measured at an

excitation of 340 nm and an emission of 460 nm. A concentration of 1.2 μM orotic acid per 1.0 mM creatinine in normal urine and 0.64 nmol orotic acid per 5.0×10^5 HeLa cells were determined by this method. The present method permitted the facile quantification of orotic acid in healthy human urine and cultured HeLa cells by spectrofluorometry and/or high-performance liquid chromatography.

In the second part of the present study, I developed an assay method for measuring DHODH activity in cultured HeLa cells and fibroblasts, and in stage III stomach cancer and adjacent normal tissues from the same patient. The assay comprised enzymatic reaction of DHODH with a large amount of dihydroorotic acid substrate, followed by fluorescence detection specific for orotic acid using the developed FL reaction in the first part of the study. The DHODH-activities in the biologically complex samples were readily measured by the assay method. The data indicate significantly higher DHODH activity in HeLa cells (340 ± 25.9 pmol/10^5 cells/h) than in normal fibroblasts (54.1 ± 7.40 pmol/10^5 cells/h), and in malignant tumor tissue (1.10 ± 0.19 nmol/mg total proteins/h) than in adjacent normal tissue (0.24 ± 0.11 nmol/mg proteins/h). This is the first report that DHODH activity may be a diagnostic biomarker for cancer.

Background

I. Orotic acid and orotic aciduria

At the beginning of the 20th century, orotic acid had been firstly chemically identified. It was recognized as a vitamin that commonly be contained in the mammalian milk at that time[1, 2]. However, with further researches focusing on orotic acid, the role of orotic acid as an intermediate of the de novo pyrimidine biosynthetic pathway had been revealed[3-5]. Moreover, excess excretion of urinary orotic acid could be observed in patients suffering from hereditary orotic aciduria[6, 7].

Hereditary orotic aciduria is an autosomal recessive disorder mainly caused by deficiency of enzyme in urea cycle. The overall incidence of these inborn errors has an overall incidence of about 1 per 9400[8]. As reported, in urines of normal persons, the concentrations of urinary orotic acid is 0.10 ~ 5.63 μmol/mmol creatinine. However, in case of patients referred to above, the concentrations of urinary orotic acid could be up to 10.9 ~ 1042 μmol/mmol creatinine[9, 10] (Table. 1).

Table 1. Reference values of urinary orotic acid of normal subjects and patients.
(Bachmann C. Eur. J. Pediatr.; Asai M. Pediatr. Int.)

	Normal subjects		Patients with orotic aciduria	
Age	N	Orotic acid (μmol/mmol creatinine)	N	Orotic acid (μmol/mmol creatinine)
Within 1 month	25	1.77 ± 0.56	3	279-1042
Within 1 year	33	2.61 ± 3.02	3	10.9-886
1-5 years	53	1.46 ± 0.87	4	18.3-440
6-10 years	47	1.32 ± 0.94	1	252
11-15 years	30	0.66 ± 0.5	2	25.3, 541
16-40 years	69	0.58 ± 0.17		
> 40 years	97	0.76 ± 0.66		

As shown in Fig. 1, in the de novo biosynthetic pathway of pyrimidine, carbamoyl phosphate is synthetized from ATP, NH_3 and HCO_3^- under the catalysis of carbamoyl phosphate synthetase 1 (CPS1), the reaction that take place in

mitochondrial of liver cells[11, 12] or is synthetized from ATP, Gln and HCO_3^- under the catalysis of carbamoyl phosphate synthetase 2 (CPS2), different from CPS1, CPS2 catalysis the reaction in cytosol of most of cells[13, 14]. Then carbamoyl phosphate combines with aspartate to form carbamoyl aspartate. After that, carbamoyl aspartate is converted to dihydroorotate by dihydroorotase. In the next step, dihydroorotate, as the substrate of dihydroorotate dehydrogenase (DHODH), is converted into orotic acid[15]. Then UMPS, a complex enzyme containing two fusions of OPRT and OMP decarboxylase, converts orotic acid into uridine monophosphate[16] (UMP). Finally, UMP is converted into other pyrimidine nucleotides.

The de novo biosynthetic pathway of pyrimidine is connected with urea cycle. Carbamoyl phosphate, the product of the first step of the pathway, also participates into the urea cycle as the substrate of OCT and reacted with ornithine to form citrulline. As reported, deficiencies in urea cycle enzymes[17, 18], particularly deficiency of ornithine transcarbamoylase (OCT), causes an overload of carbamoyl phosphate into the synthetic pathway of UMP. As a result, the concentration of orotic acid in body fluids is increased. Additionally, UMPS deficiency causes

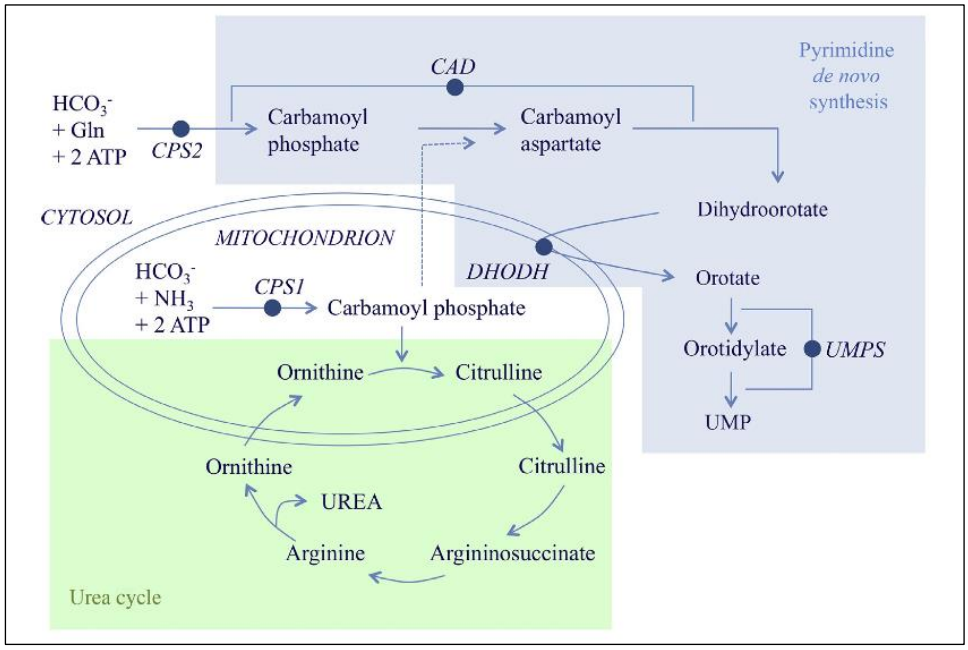


Fig. 1. Pyrimidine de novo synthesis and urea cycle.

(Source: Loffler M. J Genet Genomics, 42: 207-219 Fig. 1.)

accumulation of orotic acid in biological specimens, and finally orotic acid is excessively excreted into urines[19].

II. Diagnosis of orotic aciduria

Efforts have been paid for the diagnosis of orotic aciduria. Various methods have been developed for determination of orotic acid in bio specimens of urine and blood for the diagnosis of orotic aciduria.

Direct monitoring of urinary orotic acid without chromatographic separation has been using an ion trap mass spectrometer in negative mode[20, 21]. However, the method required pre-treatment of urine to avoid influence from matrix suppression. Another method used ^{1}H NMR spectrum. In the method, orotic acid was distinguished by a singlet resonance at 6.22 ppm. Few of other bio-substance shows same singlet resonance as same as orotic acid[22].

Chemical derivatization of orotic acid to barbituric acid, an orange product that has absorbance at 480 nm, has been applied for the colorimetric determination of urinary orotic acid. However, orotic acid must be separated chromatographically ahead of the derivatization[23-26]. Methods of enzymatic assay have been performed using either UMPS[27, 28] or DHODH[29]. In the assay, Orotic acid was catalyzed to UMP that has absorption at 295 nm or produced by DHODH followed by monitoring at 282 nm. However, these enzymatic assay methods of urinary orotic acid were easily interfered with other bio-substance thus require specific conditions.

To decrease the interference from background noise given from bio substances, chromatographic methods were developed and widely used so far. In a HPLC method coupled with UV detection, orotic acid is monitored at 280 nm. Due to its early elution and co-eluted with other urinary substances, switching columns system was established. Fraction containing orotic acid eluted from the first reverse-phase column would be than automatically delivered to the second column for further separation[30, 31]. Although orotic acid can be separated for determination, the total time for the assay is prolonged. Determination of orotic acid of a single sample could take 40 min[6, 7, 30, 31].

A chemical derivatization method coupling with HPLC detection of orotic acid has been developed aiming of using a single column. Although the equipment was simplified, the long total time for a single detection is remaining a problem due to complex procedure of sample preparation[32]. Another method using GC-MS coupled with isotope dilution and derivatization of urine residues was developed. The standard orotic acid was labeled with ^{15}N at position of 1 and 3. Trimethylsilyl derivatives were formed from urinary organic acid after extraction and were injected to GS-MS[33]. The method offered a relative accurate determination of urinary orotic acid compared with previous detections. However, it requires the specific and expensive equipment.

Capillary electrophoresis has provided a considerable selectivity of orotic acid. Under the optimized condition, the urine sample can be injected to capillary without treatment but the total analysis time is prolonged considering the process including cleanup of off-line and pre-concentration techniques[34]. Or pretreatment of urine by a reverse-phase C_{18} column could allow quick elution of orotic acid through its competitive inhibitors that would inhibit orotic acid interacting with column[35].

In a word, there is still no satisfactory detection method for orotic acid. The developed methods are either time consuming or requiring expensive equipment. Thus, for the screening of orotic aciduria, a facile and high-throughput determination of urinary orotic acid is expected.

Fluorometric methods used in determinations have showed advantages of their specificity and sensitivity compared with UV detections. However, there has been no report in which a fluorometric determination of orotic acid was introduced so far.

III. Dihydroorotate dehydrogenase

DHODH catalyzes the forth step of the de novo biosynthetic pathway of pyrimidine. Under the oxidation of DHODH, dihydroorotate is converted into orotic acid. The enzyme is located on the inner membrane of mitochondrial in most of eukaryotic

cells. The important role of this enzyme has been regarded through various fields including researches of anti-rheumatoid arthritis, antimalarial, antiviral and

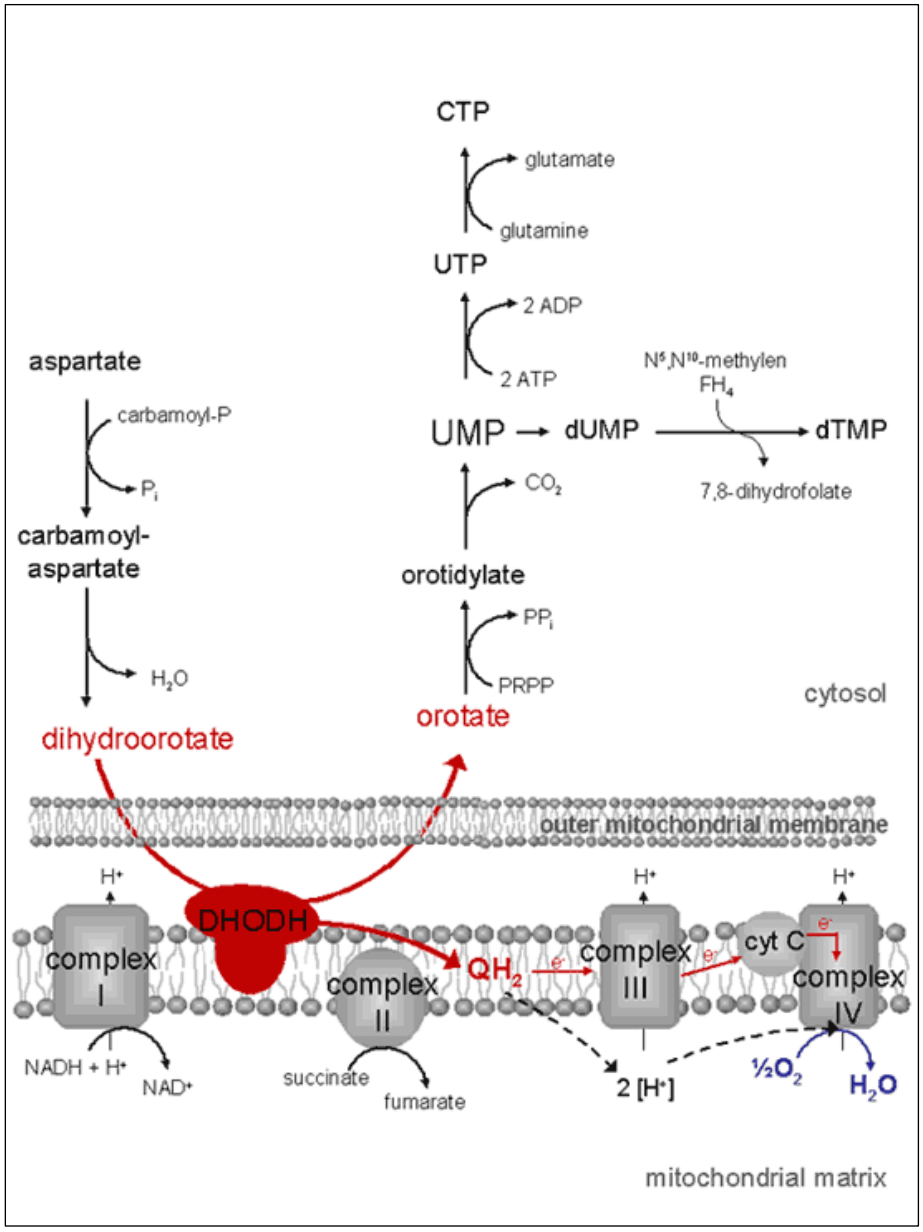


Fig. 2 Location and function of DHODH in the de novo biosynthetic pathway of pyrimidine. (From website: http://www.metabolic-database.com/html/dhodh.html)

anticancer.

In 1995, as an approved drug for the treatment of rheumatoid arthritics, leflunomide has been reported to be the inhibitor of DHODH for the first time[36-38]. In vivo and in vitro studies have revealed that leflunomide turns out to be a

prodrug and is activated in plasma to its active form, teriflunomide, for the inhibition of DHODH[39]. Further studies have shown the potential effect of leflunomide/teriflunomide in aspects of treatment of multiple sclerosis[40], dermatological diseases such as psoriasis[41], anti-virus proliferation[42-45] and anti-malaria[46-50]. In the following decades, extensive studies on relationship between structures of analogs of teriflunomide and their activities have been reported[51-55].

Another group of inhibitors, brequinar and its analogs, was studied for their anticancer properties[56-58]. Although clinical trial for orotic acid as an anticancer subject showed a modest inhibition on some solid tumors and was unfortunately terminated at phase II[59], its anticancer property through inhibition of DHODH is of significance.

The inhibition of DHODH leading to a decreased synthesis of pyrimidine since DHODH is the rate-limiting enzyme of de novo synthetic pathway of pyrimidine. Thus, rapid proliferation of cells that rely on increased concentration of pyrimidine for the synthesis of DNA is disrupted. For example, cancer cells, virus as well as parasites in an infected host cell, they require large amount of pyrimidine for their division, replication and growth. The inhibition of DHODH, however, would not affect normal function of host cells. The reason is that in most of normal host cells, pyrimidine required for cell growth is synthetized through salvage pathway of pyrimidine (Fig. 3), since de novo pathway is energetically expensive than salvage pathway[60-62]. The production of pyrimidine by salvage pathway is adequate for normal cells thus inhibition of DHODH and dysfunction of the de novo synthetic pathway of pyrimidine would do much less inhibition to normal cells than cancer cells, virus or parasites. Additionally, it has been reported that infection of virus and parasite would cause a disruption to the normal control on salvage pathway and synthesis of pyrimidine in human[60,63].

Taken all together, these studies have pointed an important role for DHODH as a target for treatment of infectious malaria and/or virus, as well as cancers. For screening of more effective synthetized drugs, researches have been using a colorimetric assay for the enzymatic activity of DHODH. In the assay, 2,6-dichloroindophenol (DCPIP) was used as the color indicator and was added to the

enzymatic reaction mixture[64, 65]. During the enzymatic reaction, dihydroorotate was oxidized to orotic acid and a pair of electrons would be released and accepted by DCPIP. After DCPIP is reduced, it turns color from blue to colorless. The method has been used for all high-throughput assays through the past decades. Values of IC50 of the synthetized inhibitors were monitored and compared. It is necessary to mention that all of DHODH used in the assay were recombinant proteins and were purified before use[66]. As shown in Fig. 2, complex III of respiratory chain that locates inside mitochondrial would receive the released electrons in ahead of DCPIP and therefore interferes with the reduction of DCPIP. In a published paper, the authors added KCN, as the inhibitor of complex on respiratory chain, into the enzymatic reaction to avoid the inhibition from complex III[67].

Since it was developed in 1970s, the colorimetric assay for DHODH using DCPIP has become the mainstream method due to its facility, despite the limitation of this method. As a result, the activity of DHODH in cells or tissues was rarely reported.

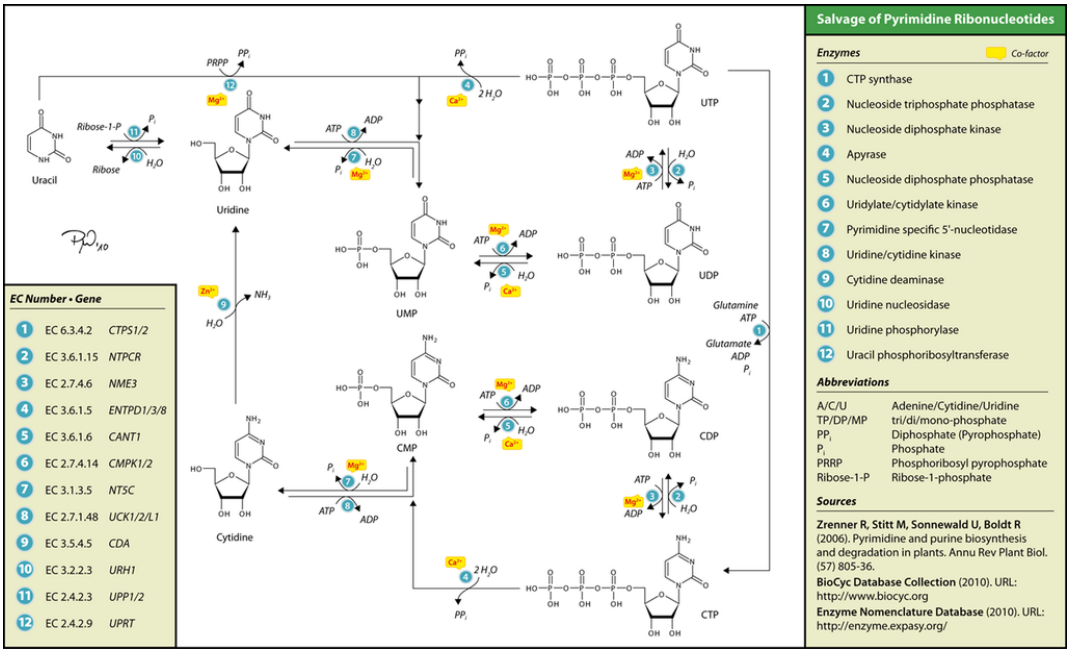


Fig. 3 Salvage pathway of pyrimidine.
(From website: https://en.wikipedia.org/wiki/Nucleotide_salvage)

IV. Aim of this project

In summary, urinary orotic acid is excessively excreted in urine of patients with orotic aciduria and thus is used as a marker for diagnosis. However, currently developed detection method for orotic acid is limited in following aspects: either a long time determination of a single sample or expensive equipment is required. There has not been a facile detection method developed for a high throughput screening of urinary orotic acid so far.

Besides, orotic acid is an intermediate in the de novo biosynthetic pathway of pyrimidine. It is produced by enzyme named DHODH, the rate-limiting enzyme of the whole pathway. Inhibition of DHODH has showed benefits in antimalarial, antivirus and anticancer properties without destroying normal function of host cells. These results indicated an abnormal activity of DHODH in these infected cells or cancer cells. However, there were no evidences of the proposed abnormal activity of DHODH due to the lack of proper method for the assay of activity of endogenous DHODH in these cells. Therefore, in this project I:

I. Developed a FL reaction for the specific determination of orotic acid in a urine, lysate of cell and tissue.

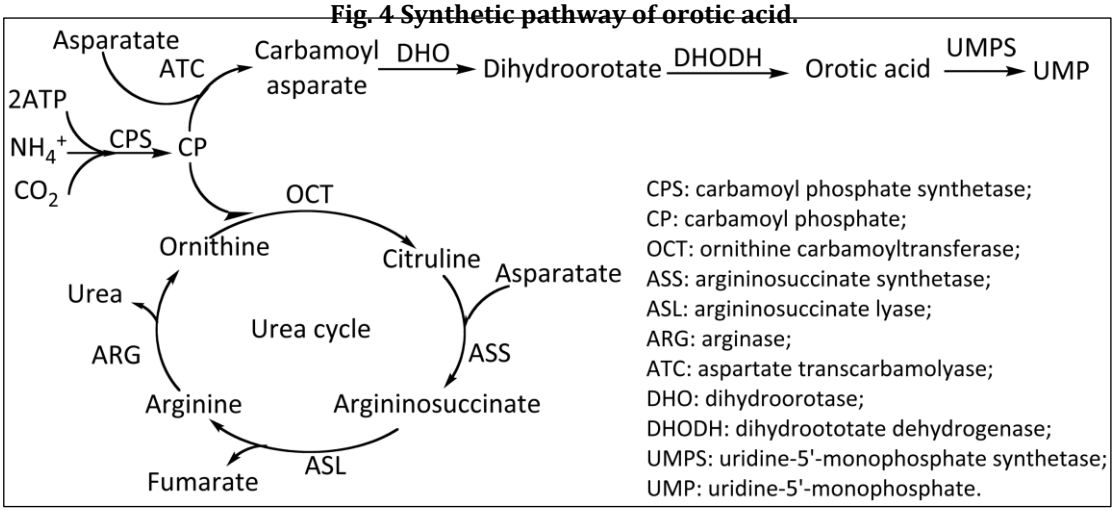
II. Established a fluorometric assay for DHODH based on the FL reaction specific for orotic acid and compared specific activity of endogenous DHODH in normal cells/tissue with cancer cells/tumors.

Chapter 1 Development of a FL reaction for the sensitive and selective determination of orotic acid in biological specimens

1.1 Introduction

Orotic acid is an intermediate in the de novo biosynthesis of UMP. This pathway starts from the formation of carbamoyl phosphate, which is used for UMP synthesis and the urea cycle[5, 7] as shown in Fig. 4. Deficiencies in urea cycle enzymes such as ornithine carbamoyltransferase and argininosuccinate synthetase, results in an overload of carbamoyl phosphate into the UMP pathway[17, 18]. In turn, an increase in the concentration of orotic acid in body fluids is observed. Additionally, a deficiency of UMP synthase causes accumulation of orotic acid in biological specimens, and thus orotic acid is intensively excreted in urines of patients suffering from hereditary disorders such as orotic aciduria and hyperammonemia[19].

Fig. 4 Synthetic pathway of orotic acid.



Determinations of orotic acid in biologically complex samples using conventional methods generally require sophisticated instrumentation for the aim of complete separation before detection of biogenic orotic acid from many

other biogenic substances. For example, column-switching HPLC with UV absorption detection[30, 31], and mass spectrometry combined with HPLC[32] or gas chromatography[33] using stable isotope-labeled orotic acid as the internal standard, are applied for quantification. Thus, a facile and inexpensive analytical method is required for the quantification of orotic acid in biological specimens.

Fluorescence (FL) is widely used for the sensitive and selective determination of biological specimens. Herein, I describe the establishment of a novel FL-derived reaction specific for orotic acid. The reaction was conducted by heating orotic acid with 4-TFMBAO, $K_3[Fe(CN)_6]$, and K_2CO_3 in an aqueous solution at 80 °C for 4 min, followed by spectrofluorometric quantification of orotic acid in human urine and cultured cells, and the accuracy of spectrofluorometric quantification is confirmed by chromatographic quantification. The presented method is of facility, selectivity and sensitivity for the determination of biogenic orotic acid.

1.2 Materials and methods

1.2.1 Reagents

Chemicals obtained from commercial supplies were used without further purification. Orotic acid was obtained from TCI (Kyoto, Japan). 4-TFMBAO and other benzamidoxime (BAO) analogs were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of analytical or guaranteed reagent grade. H_2O was purified by a Milli-Q system and used for the experiments. Orotic acid and its related bio-substances, 4-TFMBAO and its analogs, $K_3[Fe(CN)_6]$ and K_2CO_3, were weighed in each flask or polypropylene tube (Japan Eppendorf, Tokyo, Japan) and then dissolved in water.

1.2.2 Preparation of urine and lysate of HeLa cells

A urine sample was collected from one healthy donor (male, 25 years old) and centrifuged at 3500 × *g* for 15 min. The supernatant was stored at −40 °C. Then, the urine specimen was diluted in water to 5 % (v/v) before analysis. Creatinine concentration (8.85 mM) in the undiluted urine was measured to normalize the

rate of kidney filtration using a colorimetric assay kit (AUTION Sticks) purchased from ARKRAY Factory Inc. (Shiga, Japan).

HeLa cells (1.0×10^7) were cultured, collected and then suspended in 4.0 ml of H_2O. The cells were sonicated in an ice bath for 30 min followed by centrifugation at 3500 × g for 5 min. The supernatant was collected for analysis.

1.2.3 FL reaction of orotic acid in urine or cells

A portion (200 µl) of the biological specimen of 5% urine or cell lysate (5.0×10^5 cells) was mixed with 50 µl of 0, 10 or 20 µM standard orotic acid. The sample solution (250 µl) was mixed with 250 µl of 4.0 mM of 4-TFMBAO, 250 µl of 8.0 mM $K_3[Fe(CN)_6]$ and 250 µl of 80 mM K_2CO_3. The mixture (1.0 ml) was then heated at 80 °C for 4.0 min, followed by cooling in an ice bath for ca. 2.0 min to stop the reaction.

1.2.4 FL measurement by spectrofluorometry

The relative FL intensity produced by the reaction with 4-TFMBAO was measured with an FP-6300 spectrofluorometer (Jasco, Tokyo, Japan) at maximum excitation and emission wavelengths of 340 nm and 460 nm, respectively. For the reactions with other BAO analogs, the wavelengths at each maximum of excitation and emission were set at 310–340 nm and 410–460 nm, respectively. Bandwidths were set at 5 nm for both the excitation and emission.

1.2.5 HPLC measurement

A reversed-phase HPLC system was employed, consisting of a quaternary gradient pump (PU-2089 Plus; Jasco), a reversed-phase C_{18} column (COSMOSIL 5C18-AR-II, 4.6 mm i.d. × 150 mm; Nacalai Tesque, Kyoto, Japan), a UV absorption spectrometer (UV-2070 Plus; Jasco), and a spectrofluorometer (FP-2020 Plus, Jasco). The separation was carried out using 40% (v/v) methanol in H_2O as the mobile phase. The flow rate was 1.0 ml/min. The column elute was monitored at 460 nm (emission) and 340 nm (excitation) for FL detection, and 280 nm (absorption) for UV detection.

1.2.6 Analysis of FL product of orotic acid by MS

Orotic acid (1.0 mM) was reacted according to the above-described procedure for the FL reaction with 4-TFMBAO. The FL product in the reaction mixture was separated and collected by reversed-phase HPLC using a mobile phase (50% methanol in H_2O) at a flow rate of 1.0 ml/min. The FL product was observed at 5.4 min as a single peak. The separation (100 µl each per injection) was repeated 24 times. The collected fractions were evaporated to remove methanol, and then lyophilized. A pale blue powder of the product was obtained and dissolved in 150 µl of methanol. The product in the solution was analyzed by a negative electrospray ionization mass spectrometer (ESI-MS; JEOL Ltd., Tokyo, Japan).

1.3 Results and Discussion

1.3.1 Investigation of FL reaction conditions

Previously I have developed two FL reactions specific for uracil[68] and cytosine[69] using BAO and 4-TFMBAO as the fluorogenic reagent, respectively. These reactions were conducted by heating in a strongly alkaline KOH solution. In this study, the FL reaction for orotic acid was prepared by heating with 4-TFMBAO in a weakly alkaline K_2CO_3 solution in the presence of $K_3[Fe(CN)_6]$ as an oxidant. To specifically yield a FL product from orotic acid, I firstly investigated different concentrations of K_2CO_3, 4-TFMBAO and $K_3[Fe(CN)_6]$ by using uracil and cytosine as control compounds and then investigated the reaction time and temperature as well (Fig. 5).

As shown in Fig. x, when the final concentrations of K_2CO_3 were 20–30 mM in the reaction mixture (Fig. 5A), the maximum and selective FL production from orotic acid was obtained. As a result, a final concentration of 20 mM K_2CO_3 in the FL reaction mixture was employed for the typical procedure.

By using 20 mM K_2CO_3, 1.0 mM 4-TFMBAO and 2.0 mM $K_3[Fe(CN)_6]$, I earned the maximum FL intensity from orotic acid. A reaction time between 1.0 and 5.0 min at 80 °C performed an increasing FL intensity for orotic acid, however, a 4.0 min reaction time provided best selectivity for orotic acid.

In Fig. 6, FL excitation and emission spectra of the FL product obtained from orotic acid at final concentrations of 1.0 2.0, 5.0 and 10 μM in the FL reaction mixture were shown. The maximum wavelengths were 340 nm for excitation and 460 nm for emission, respectively. The FL intensities were proportional to the final concentrations of orotic acid in the FL reaction mixture.

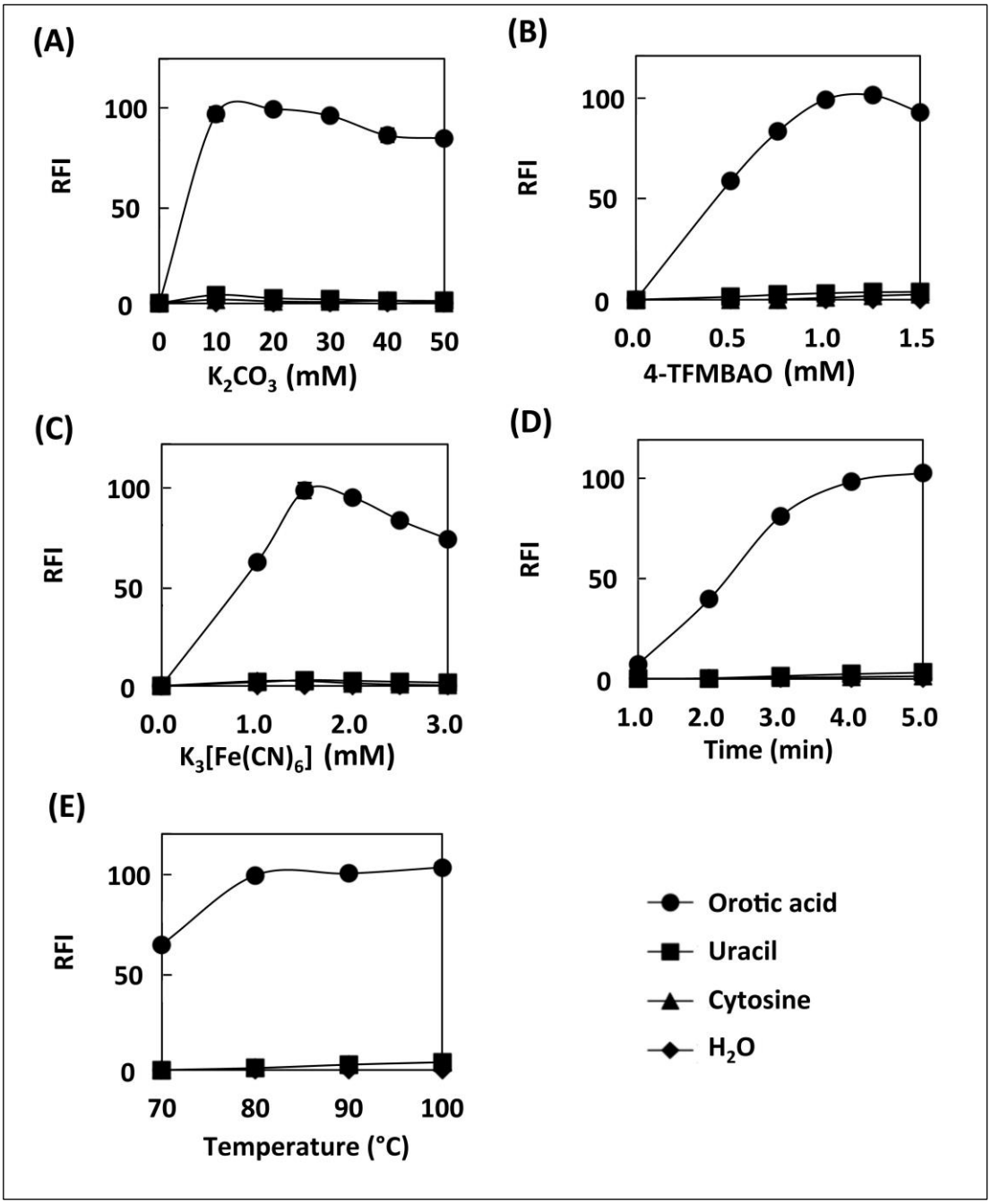


Fig. 5 Optimization of reaction conditions for the selective FL reaction for orotic acid.

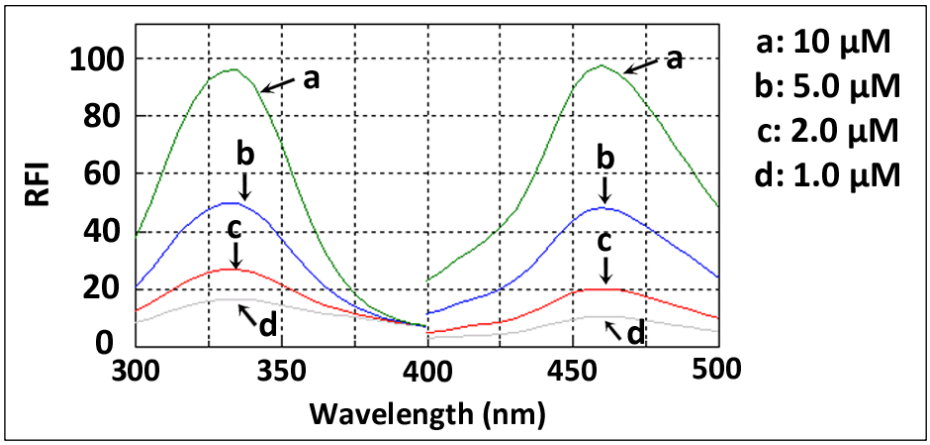


Fig. 6 FL excitation and emission spectra from orotic acid.

Various analogs of BAO were investigated for the FL reaction of orotic acid,

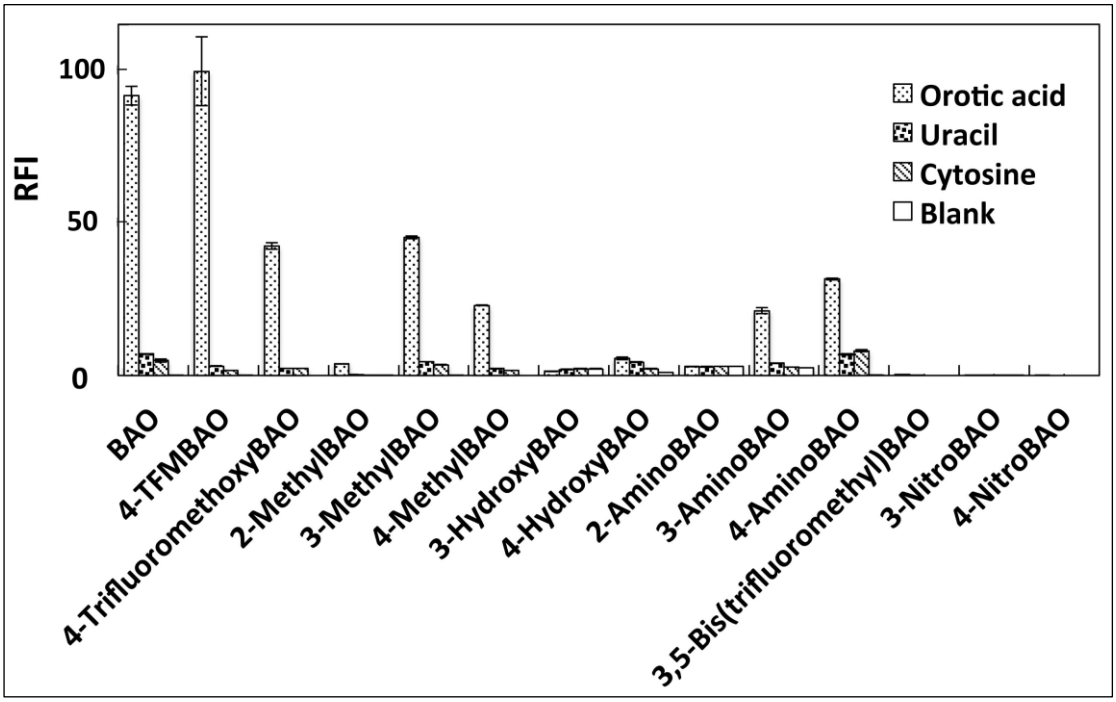


Fig. 7 FL intensity obtained from orotic acid, uracil and cytosine with various BAO analogs.

uracil and cytosine, under the optimized reaction conditions (Fig. 7). As a result, among all the analogs of BAO, 4-TFMBAO as a fluorogenic reagent was the most selective and intensive for orotic acid, despite that BAO, 4-trifluoromethoxyBAO, 3-methylBAO, 4-methylBAO, 3-aminoBAO and 4-aminoBAO provided FLs for

orotic acid. However, 2-MethylBAO, 3-hydroxyBAO, 4-hydroxyBAO, 2-aminoBAO, 3,5-bis(trifluoromethyl)BAO, 3-nitroBAO and 4-nitroBAO gave negligible FLs for all tested analytes.

1.3.2 Specificity of the FL reaction for orotic acid

Specificity of the present FL reaction for orotic acid was studied by using 62 kinds of bio-contents that might occur in biological specimens (Fig. 8). The strongest FL intensity was earned by orotic acid under the optimized conditions. Negligible FL intensities compared with the reagent blank (H_2O) were detected with uracil, cytosine, folic acid, thiamine and pyridoxine. Compounds including other analogues of orotic acid (dihydroorotic acid, 1-methyluracil, 5-fluorouracil, 6-methyluracil, 5,6-dihydrouracil, 5-methylcytosine, thymine, adenine and guanine), nucleosides (uridine, pseudouridine, cytidine, thymidine, adenosine and guanosine), nucleotides (5′-UMP, 5′-CMP, 5′-TMP, 5′-AMP and 5′-GMP), 20 kinds of amino acids, vitamins (D-pantothenic acid, L-ascorbic acid, biotin, riboflavin, nicotinic acid and nicotinamide), other bio-contents (bilirubin, uric acid, creatine, creatinine and urea) and sugars (sucrose, glucose, fructose, lactose and ribose) were not detected with any FL. On the basis of the presented results, it was indicated that the FL reaction is capable of being applied for the specific quantification of orotic acid in biological specimens.

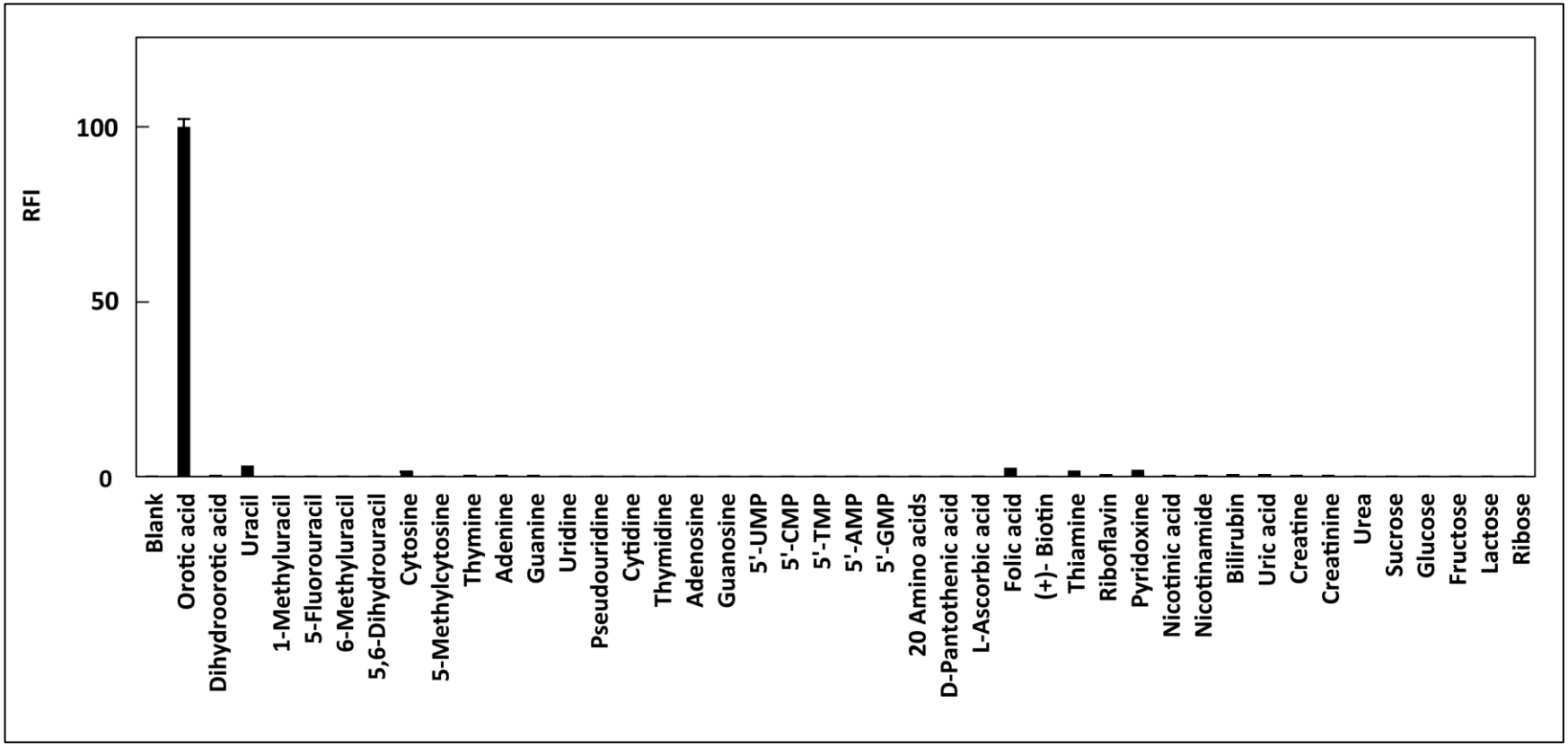


Fig. 8 FL production from orotic acid and other related bio-substances under the optimized reaction conditions for 4-TFMBAO.

1.3.3 Investigation of reaction mechanism

A proposed mechanism for the FL product of the reaction between orotic acid and 4-TFMBAO was shown in Fig. 9. I previously reported that FL reaction with BAO in solution containing KOH that provided a strong alkaline condition was selective for uracil rather than for orotic acid. Herein I found that under weakly alkaline conditions and being heated in the presence of oxidant, the carboxyl group of orotic acid was possibly firstly eliminated followed by the reaction between carbanion of orotic acid and the amidoxime moiety of 4-TFMBAO. Based on the results shown in Fig. 9, substitution at the position of either *N*1 or *C*5 disturbed the FL product to be formatted. This explained that no FL would be yielded by nucleosides and nucleotides without carboxyl group.

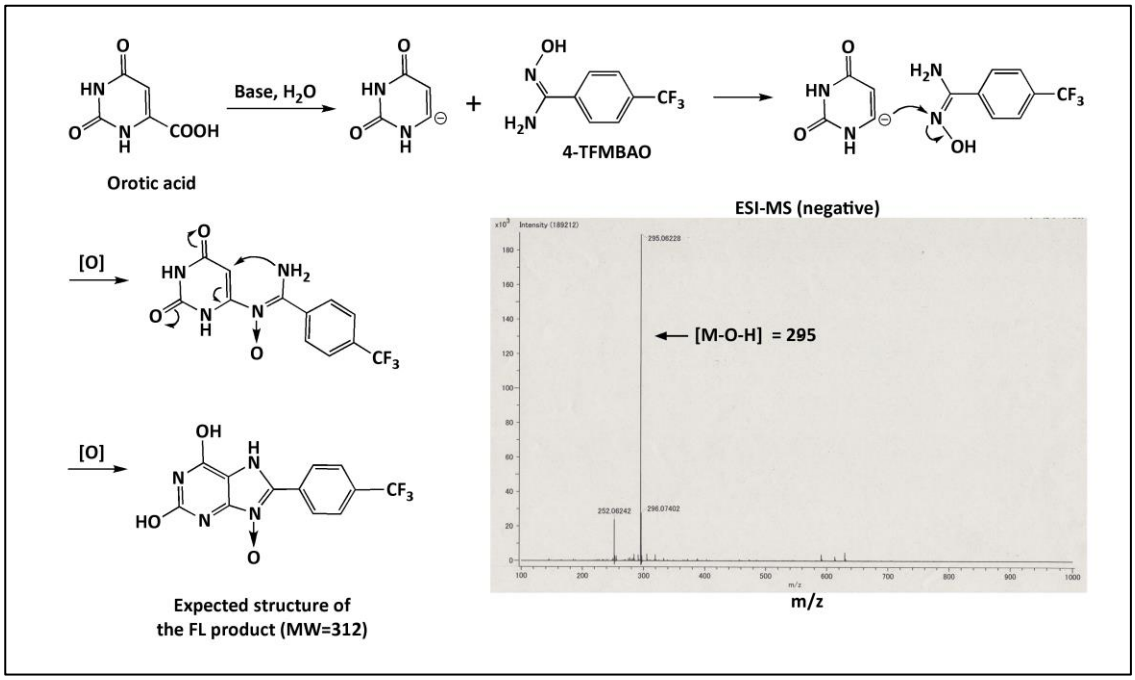


Fig. 9 Plausible reaction mechanism for the FL reaction of orotic acid with 4-TFMBAO, $K_3[Fe(CN)_6]$ and K_2CO_3 in an aqueous solution.

The FL product was separated and collected by reversed-phase HPLC, followed by analysis using ESI-MS. In Fig. 9, the MS spectrum showed an ion peak at m/z = 295 that was probably corresponding to the [M-oxygen-H]-ion, since *N*-oxide compounds were reported to inhibit a strong peak of [M-oxygen] ion in MS

spectra[70, 71]. Thus, it was suggested that the FL product plausibly was a phenyl-substituted purine derivative (Fig.9).

1.3.4 Quantification of orotic acid in urine and cell lysate by the present FL reaction

I quantified the concentrations of orotic acid in the urine from a healthy adult (male) as well as the lysate of cultured HeLa cells by using HPLC (Fig. 10 & Fig. 11 A) or spectrofluorometry (Fig. 11 B). Results of these two methods were confirmed by each other and they agreed with the same concentrations of orotic acid in tested specimens. In Fig. 11, the standard addition assay was performed for the quantification of orotic acid in the biological specimens referred to above. As shown in Fig. 10, I used reversed-phase HPLC coupled with FL and UV detectors. It provided separation and detection of orotic acid in both of diluted urine and lysate of HeLa cells as the FL product after the reaction with 4-TFMBAO.

According to the peak of FL product under the monitoring of FL detector, retention time of the FL product of orotic acid was 9.6 min. However, at the same retention time, there was no UV absorption peak of the FL product under the detection of UV (280 nm). The result suggested a much lower sensitivity of the UV detection compared with the FL detection.

I also attempted to detect orotic acid in the specimens without the FL reaction by HPLC. However, without a column switching system, orotic acid in the specimens would co-eluted with other biogenic compounds rapidly at 3.5 min included in the dead volume of the column.

Standard orotic acid (2.5 and 5.0 μM) was added to each specimen for the quantification of concentrations of orotic acid in the diluted urine sample and lysate of HeLa cells.

Using the method of HPLC coupled with FL detection (Fig. 11 A), the concentrations of endogenous orotic acid were 0.56 ± 0.02 μM in 5% urine and 3.35 ± 0.20 μM in the lysate of HeLa cells (5.0×10^5 cells in 200 μl), respectively. The linear equations (Y = FL intensity, X = μM added standard orotic acid, R^2 = linear regression) between concentration of added standard orotic acid to diluted

urine (Y = 8.6X + 4.8333, R^2 = 0.99124) or lysate of HeLa cells (Y = 3.58X + 11.983, R^2 = 0.9901) and earned FL intensities were shown in Fig. 11. Both of the quantification curves earned good linearity.

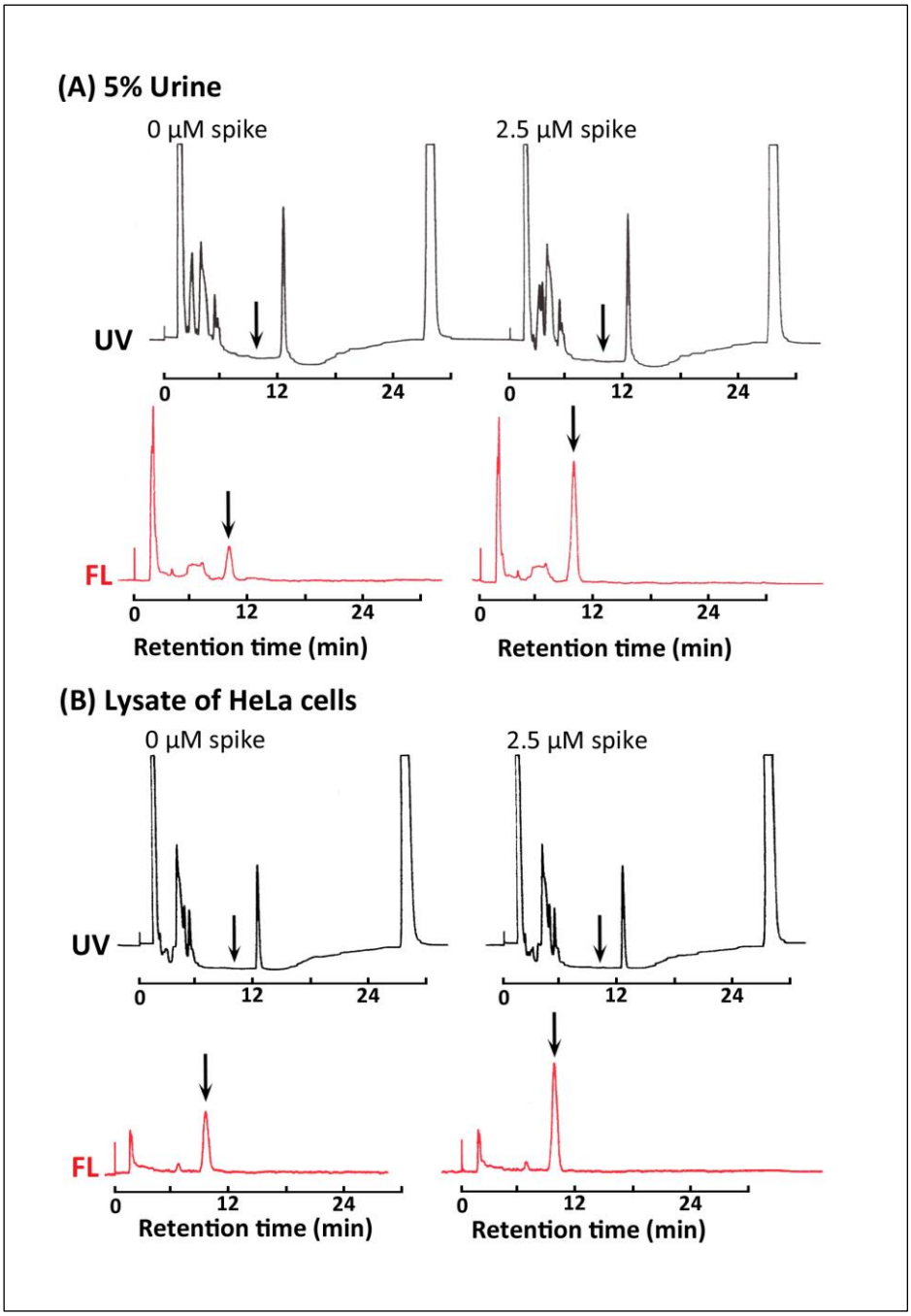


Fig. 10 Quantitative determination of orotic acid in urine (A) and HeLa cells (B) by HPLC with FL and UV detection after the FL reaction with 4-TFMBAO.

For the determination of orotic acid by spectrofluorometry alone, the FL intensities resulted from the full reactions of the urine and cell lysate specimens should be firstly subtracted by the background FL intensity earned from the blank reaction. The blank reactions were conducted as same as full reactions, except that $K_3[Fe(CN)_6]$ was eliminated. This background correction can distinguish the presence of endogenous orotic acid from other FL substances including vitamins and/or oxidized by-products in the specimens.

By spectrofluorometry, the concentrations of endogenous orotic acid in the 5% urine sample and in the cell lysate (5.0×10^5 cells in 200 µl) were 0.55 ± 0.01 µM and 3.26 ± 0.14 µM, respectively (Fig. 11 B). The linear equations of the standard addition curves were $Y = 43X + 23.833$ ($R^2 = 0.9974$) for the urine and $Y = 18.8X + 61.333$ ($R^2 = 0.99985$) for the cell lysate.

The concentrations of the endogenous orotic acid obtained by the HPLC and spectrofluorometric methods were in accordance with each other. Concentration of creatinine was detected for the normalization of urinary orotic acid during the calculation of concentration. The concentration of urinary orotic acid in the tested urine sample was 1.2 µM per 1.0 mM creatinine, which agreed well with the concentration reported previously[9]. As a result of quantification of endogenous orotic acid in HeLa cells, 0.64 nmol of orotic acid was detected in 5.0×10^5 of total HeLa cells. The precision accuracy of both the methods was estimated by the repeated (n = 5) determinations within a day.

I studied the relative standard deviation, lower detection limit as well as recovery of endogenous orotic acid for presented FL reaction. The values of relative standard deviation for the urine and the lysate of HeLa cells were 4.4% and 5.9% by HPLC method. In case of spectrofluorometry method, the values of relative standard deviation for the urine and the lysate of HeLa cells were 2.2% and 4.4%, respectively. The lower detection limits of standard orotic acid in the FL reaction mixture were approximately 1.0 nM by the HPLC method and 0.1 µM by the spectrofluorometric method. The fluorescent signals were at least three fold higher than those of the base-line noise for the HPLC or the reagent blank for the spectrofluorometry. The recovery of the endogenous orotic acid was 75 % for

the urine, and 35 % for the lysate of HeLa cells (5.0 ×10^5 cells in 200 μl) compared with the calibration standards in the absence of the biological specimens.

The FL reaction of endogenous orotic acid with 4-TFMBAO might be influenced

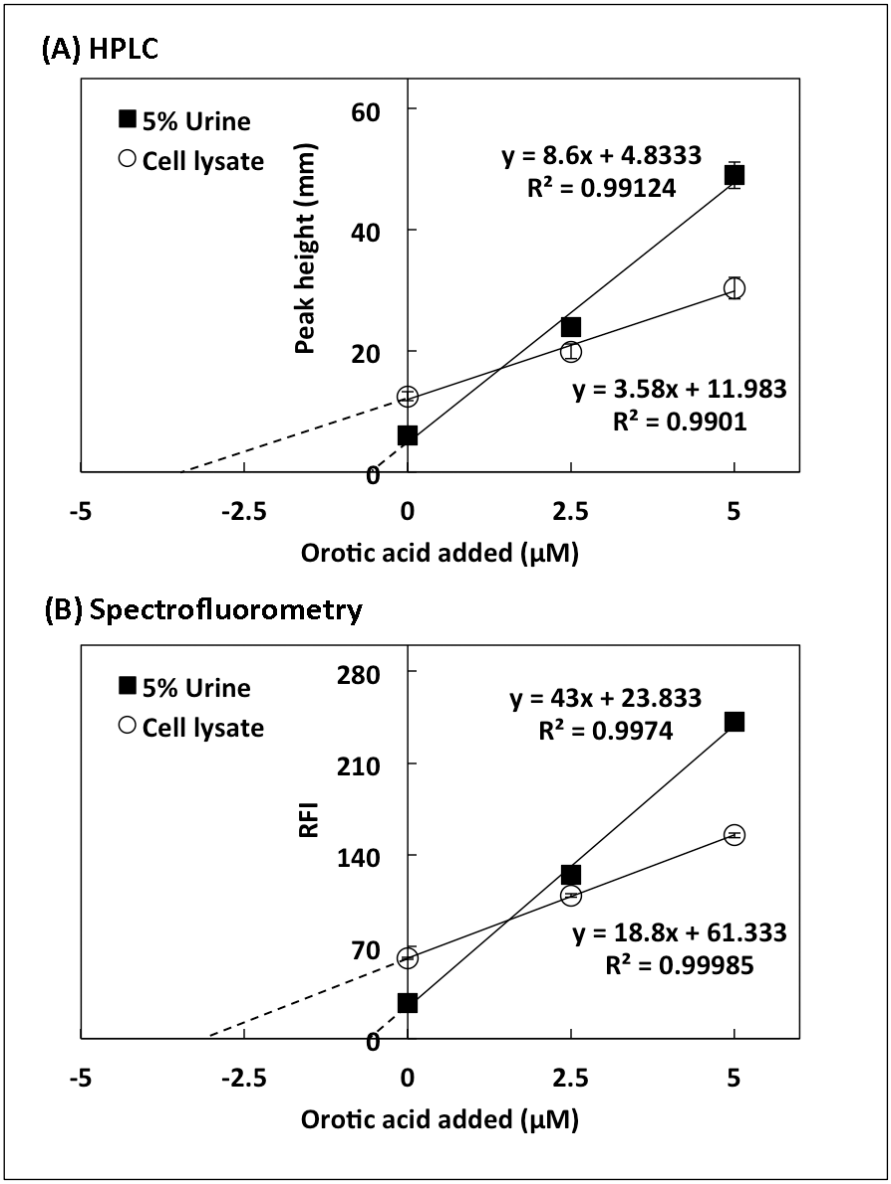


Fig. 11 Linear equations of the standard addition curves for diluted urine and lysate of HeLa cells by HPLC (A) and spectrofluorometry (B).

by the large amount of other bio-substances, because the FL production from orotic acid was inhibited by the bio-matrices in the cell lysate. However, the FL signals were proportionally increased with increasing concentrations of orotic acid spiked in the cell lysate, providing a good linear regression of R^2 of 0.9901–

0.99985 as shown in Fig. 11. To avoid the inhibition of the FL reaction, the specimens should be diluted with water as possible. When the cell lysate was twice diluted to 2.5 × 10^5 cells in 200 μl of H_2O, this treatment increased the reaction recovery by approximately two folds.

1.4 Conclusion

In this study,Ideveloped for the first time a novel fluorescent reaction method that provides a rapid fluorescent derivatization of orotic acid using 4-TFMBAO as a fluorogenic reagent. This reaction was specific toward orotic acid among 62 kinds of bio-substances tested (Fig. 8). Thus, this reaction could be readily applied to FL-based HPLC method for the sensitive determination of biogenic orotic acid in healthy human urine and cultured HeLa cells. In the presence of high concentrations of uracil and cytosine, however the 4-TFMBAO reaction gave an increased FL intensity for orotic acid since the 4-TFMBAO reagent slightly reacted with uracil and cytosine, and their FL products eluted at almost the same retention time as that for the FL product of orotic acid in the FL-based HPLC. When 1.0 μM orotic acid in the presence of 10 μM uracil or 10 μM cytosine was reacted with 4-TFMBAO, the FL-peak height of orotic acid was increased by 25 and 10 %, respectively. The co-elution of those FL products might result in a large value of the endogenous concentration of orotic acid in patient's urines especially that contain intensively higher concentrations of uracil and/or cytosine than that of orotic acid[70]. Therefore, the present FL-based HPLC method permits the determination for the endogenous orotic acid in biological specimens containing low concentrations of uracil and cytosine.

However, the methods described herein provide advantages in terms of facility and sensitivity. For example, the entire assay time including the sample preparation of the endogenous orotic acid was approximately 20 min by the spectrofluorometric method and 40 min by the HPLC method. These assay times are much shorter than those of conventional methods (50–120 min) such as column-switching HPLC with UV detection and chromatographic MS using stable

isotope-labeled orotic acid as an internal standard. Although the HPLC methods coupling UV detection can afford a wide linear range for the calibration of orotic acid from approximately 1.0 to 1000 μM in the sample, this sensitivity is approximately one tenth lower than that of the present FL-based HPLC method. My FL-based HPLC method provided a determinable range from 0.1 to 100 μM orotic acid in the reaction mixture. Concentration of orotic acid higher than 100 μM caused saturation for the FL production. In this case, samples should be diluted for the F00L reaction. Consequently, the present FL-based method is readily operable and fairly rapid because no pre-treatment was required for the biological specimens.

Chapter 2 Assay of dihydroorotate dehydrogenase as a potential cancer marker

2.1 Introduction

Dihydroorotate dehydrogenase (DHODH) is an enzyme in the uridine monophosphate biosynthetic pathway that catalyzes the oxidation of dihydroorotic acid (DHO) to orotic acid (Fig. 12). This enzyme mainly localizes to mitochondria membranes in mammal cells. Mutation of human DHODH gene is associated with a human genetic disorder. DHODH inhibitors such as leflunomide and teriflunomide are reported to be therapeutic drugs for rheumatoid arthritis and psoriasis. Several DHODH inhibitors have been also been reported to have anti-malaria, anti-viral and anti-tumor effects.

An indirect colorimetric DHODH assay method was previously developed based on 2,6-dichlorophenolindophenol (DCPIP) reduction. In this reaction, DHODH catalyzes DHO oxidation to orotic acid and DCPIP reduction, resulting in

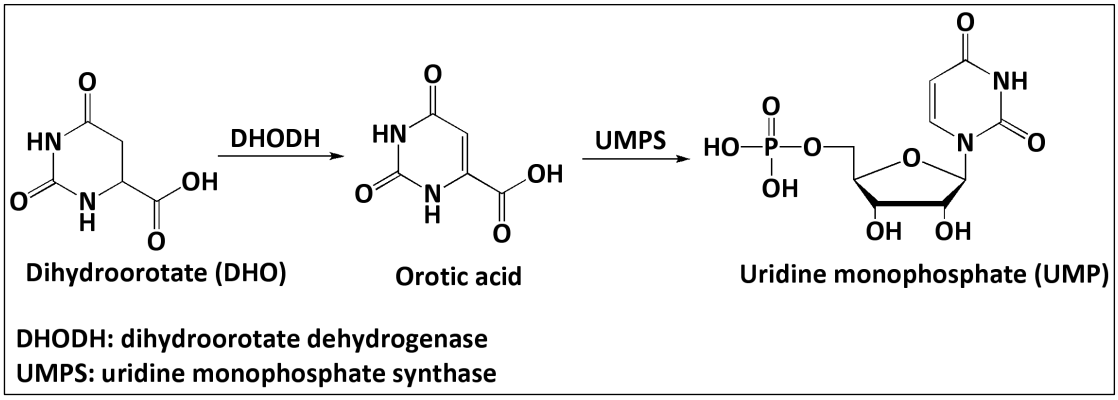


Fig. 12 DHODH is an enzyme in the de novo synthetic pathway of UMP.

a color change of DCPIP from blue to colorless that can be measured using a spectrophotometer. This method has been used to evaluate synthetic inhibitors of recombinant human DHODH. However, when used for assaying DHODH activity in biologically complex samples containing mitochondrial membranes, the

respiration chain complex in the mitochondrial membrane matrix significantly inhibited the redox reaction between DCPIP and DHO.

In chapter 1, I have established a novel FL reaction with 4-TFMBAO reagent for the specific quantification of orotic acid. This non-FL reagent provides a strong FL signal for orotic acid without interference from other biological substances. In this study, I applied this FL reaction to the assay of DHODH activity in cultured cells and in human stomach tissues. Facile, selective and sensitive FL assay of the DHODH activity was achieved by incubating DHO substrate with a small quantity of DHODH present in biologically complex samples without a need for enzyme purification.

2.2 Materials and methods

2.2.1 Chemicals

Gastric cancer tissue and its adjacent normal tissue of a stage III patient were purchased from COSMO Bio, Co., Ltd (Tokyo, Japan). Chemicals obtained from commercial supplies were used without further purification. Orotic acid and coenzyme Q10 were obtained from TCI (Kyoto, Japan). 4-TFMBAO and DHO were purchased from Sigma–Aldrich (St. Louis, MO, USA). All of the chemicals were of analytical or guaranteed reagent grade. H_2O was purified by a Milli-Q system and used for the experiments.

2.2.2 Cell culture

HeLa cells were cultured in a 10-cm culture dish in Dulbecco's modified Eagle's medium containing 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B and were incubated at 37°C. Fibroblast cells were cultured in a 10-cm culture dish in minimum essential medium-Alpha containing 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B and were incubated at 37°C. Cells were collect after grew to 90% confluence. They were removed from dish by trypsin. After washing with 1 × PBS, the cells were stored at -80 °C before analysis.

2.2.3 Preparation of lysate of cells and tissues

The cells were lysed in water (10^6 cells/ml) at 4 °C by sonication for 10 min. The lysates were then centrifuged at 16000 g for 20 min and supernatants were used for the enzymatic reaction and determination of endogenous orotic acid. Tumour and adjacent normal tissues from a patient with stage III stomach cancer were purchased from Tissue Solutions Ltd (Glasgow, UK). The supplier declared that the tissues have been obtained according to the legal and ethical requirements with the approval of an ethics committee and with anonymous consent from the donor or nearest relative. All experiments in this study were carried out in accordance with my university's ethical guidelines. The wet tissues were cut into tiny pieces and homogenized in a glass homogenizer (60 mg wet weight /ml H_2O) for 10 min followed by sonication for another 10 min. The homogenates were centrifuged at 16000 g for 20 min and supernatants were used for the enzymatic reaction and determination of endogenous orotic acid.

2.2.4 Enzymatic reaction condition for DHODH

Each of the lysate (300 µl) was incubated with 200 mM K_2CO_3-HCl buffer (pH 8.0), 0.2% triton x-100, 500 µM DHO and 100 µM co-enzyme Q10 in a total volume of 1.0 ml of enzymatic reaction mixture. The enzymatic reaction mixture was incubated under 37 °C for 60 min.

2.2.5 FL detection with 4-TFMBAO

The enzymatic reaction mixture (100 µl) or the lysate (100 µl) was mixed with 100 µl of 0, 0.5, 1.0µM standard orotic acid, 50 µl of H_2O, 250 µl of 4.0 mM 4-TFMBAO, 250 µl of 8.0 mM $K_3[Fe(CN)_6]$ and 250 µl of 80 mM K_2CO_3 in H_2O. The mixtures were immediately heated at 80 °C for 4.0 min, followed by cooling in an ice bath for ca. 2.0 min to stop the reaction. The FL intensity of each of the reaction mixture was measured by FP-6300 spectrofluorometer (Jasco, Tokyo, Japan). The excitation and emission maxima were 340 nm and 460 nm, respectively.

2.2.6 Normalization of sample size

The enzymatic activities and concentrations of endogenous orotic acid in the lysates of HeLa cells and fibroblast cells were normalized using cell numbers for comparison. Concentration of total proteins in the lysates of both malignant tumor tissue and its adjacent normal tissue were detected using Quick Start™ Bradford 1× Dye Reagent from Bio-Rad Laboratories, Inc. (U.S.A.). The enzymatic activities and concentrations of endogenous orotic acid in the lysates of tissues were normalized using concentration of total protein in the lysate for comparison.

2.2.7 Colorimetric assay for DHODH

Each of the lysate (300 µl) was incubated with 200 mM K_2CO_3-HCl buffer (pH = 8.0), 0.2% triton x-100, 500 µM DHO and 100 µM co-enzyme Q10 and 500 µM DCPIP in a total volume of 1.0 ml of enzymatic reaction mixture. The reaction was initiated by the addition of lysate. Absorbance of the enzymatic reaction mixture was measured at 610 nm 1 min, 30 min and 60 min after the reaction was initiated.

2.3 Results and discussion

2.3.1 Assay principle

Fig. 13 showed the principle for the enzyme assay of DHODH. The substrate, DHO was converted into orotic acid with DHODH in the enzymatic reaction in the presence of K_2CO_3-HCl (pH=8.0), triton x-100 and coenzyme Q10 at 37 °C.

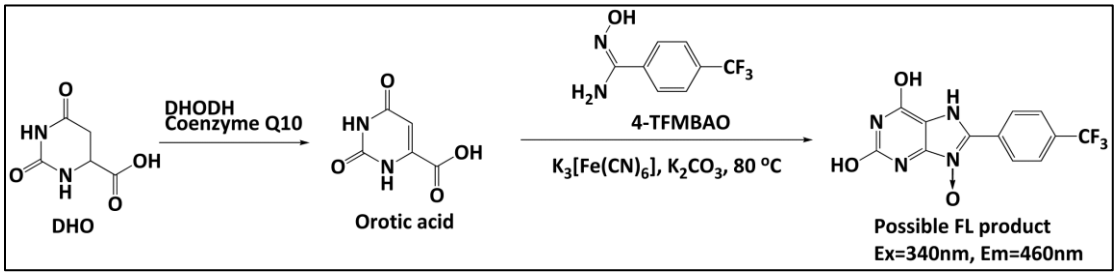


Fig. 13 Principle of the DHODH assay based on a FL reaction.

The fluorogenic reagent, 4-TFMBAO reacted with orotic acid in the enzymatic reaction mixture in the presence of $K_3[Fe(CN)_6]$ and K_2CO_3 at 80 °C for 4 min, and gave strong FL intensity for orotic acid but not for the excess substrate

DHO and neither for most of other biogenic substances such as nucleobases, nucleosides, nucleotides, amino acids, vitamins and sugars. Therefore, the assay comprised the enzymatic reaction of DHO with DHODH, followed by the FL reaction of the produced orotic acid with 4-TFMBAO.

This assay format enabled the specific assay of DHODH activity in biologically complex sample such as cultured cells and tissues, and had sufficient selectivity and sensitivity for measuring the DHODH activity.

2.3.2 Conditions for assay of DHODH activity in HeLa cells.

To optimize the assay conditions, cell lysate of was used as the enzyme source of DHODH. Reaction buffer of 50 mM, 200 mM and 250 mM K_2CO_3-HCl (pH 8.0) and

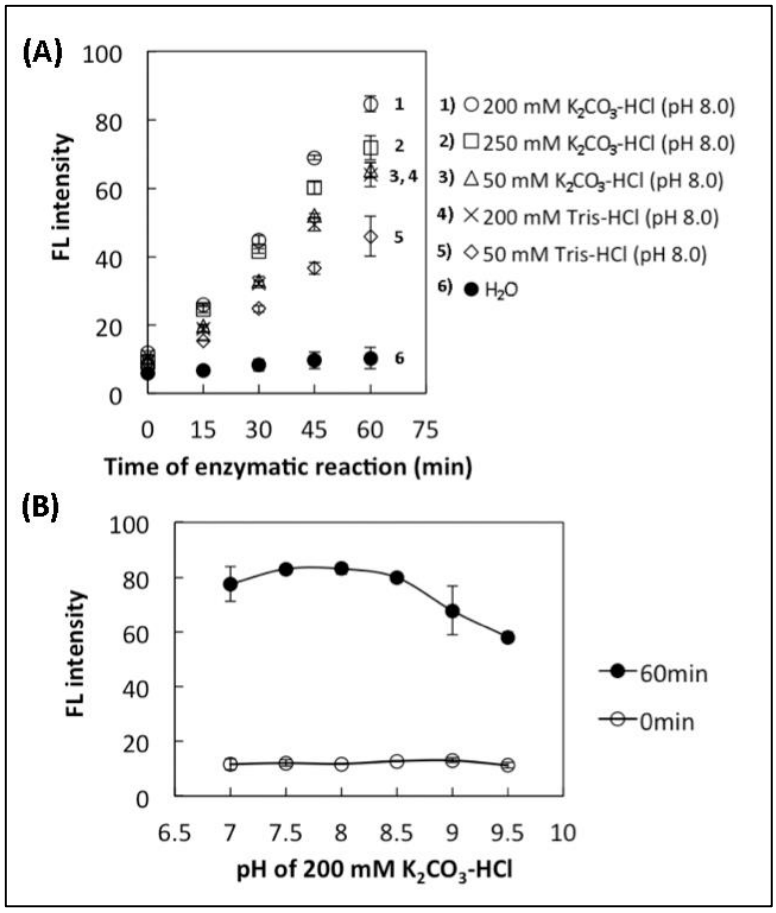


Fig. 14 Effects of buffer concentration and pH on DHODH activity.

50 mM and 200 mM Tris-HCl (pH 8.0) were investigated on the DHODH reaction (Fig. 14 A). Using 200 mM K_2CO_3-HCl (pH 8.0) (Fig. 14 A) as a reaction buffer, the

FL intensity of orotic acid was highest at a pH of 7-8.5 (Fig. 14 B). As previously reported[71], the 4-TFMBAO reagent gave a highest FL intensity for orotic acid in the presence of 10 – 40 mM K_2CO_3 (pH ca. 11). Therefore, 200 mM K_2CO_3-HCl (pH 8.0) was chosen for the DHODH reaction and a final K_2CO_3 concentration of 40 mM (pH 11) was used for the FL reaction.

2.3.3 Calculation of specific activity of DHODH in HeLa cells

I measured the specific activity of DHODH in the cultured HeLa cells. To calculate the produced orotic acid by DHODH in HeLa cells, I incubated the enzymatic reaction mixture at 37 °C for 1.0 h. Each of the enzymatic reaction (1.0 ml) was prepared in a tube and was initiated by addition of 0, 25, 125, 250 and 500 μM

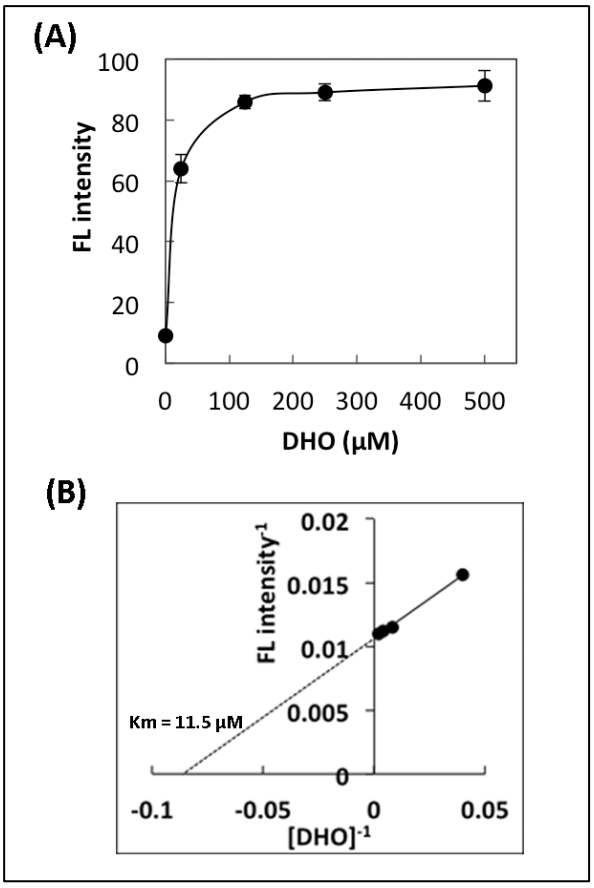


Fig. 15 Effect of substrate concentration on DHODH activity.

DHO (Fig. 15 A). Addition of sufficient concentration of orotic acid is necessary and thus a final concentration of 500 μM orotic acid was added for the enzymatic

reactions in the following study. The Km value of DHODH by my method was 11. 5 μM (Fig. 15 B). A portion of 100 μl of the enzymatic reaction mixture was directly used for the FL reaction with 4-TFMBAO at 80 °C for 4 min.

For the quantification of produced orotic acid in the enzymatic reaction mixture, the standard curve of orotic acid was made by standard addition of orotic acid to the enzymatic reaction mixture, without the incubation time. The FL

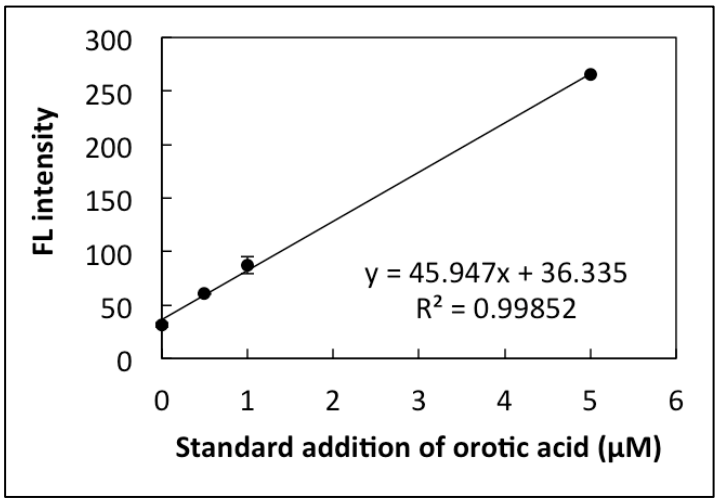


Fig. 16 Standard curve of FL intensity earned by orotic acid added to enzymatic reaction mixture.

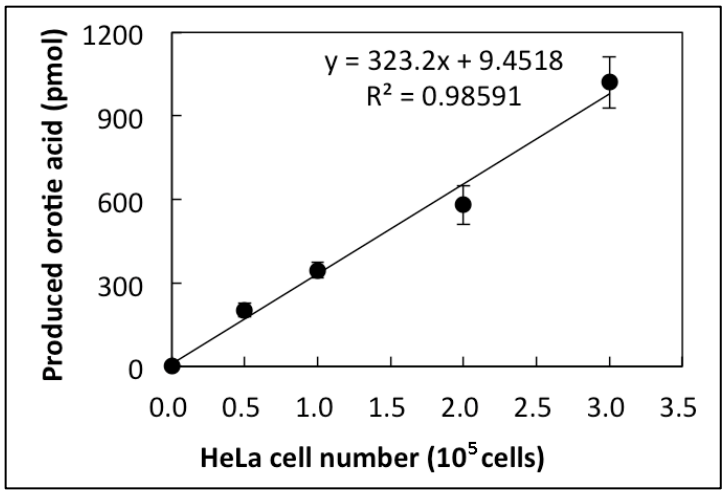


Fig. 17 Effect of HeLa cell number on DHODH activity.

intensity due to the orotic acid concentration was well proportional to the orotic acid concentration (0, 0.5, 1.0 and 5.0 μM). A weak FL intensity without the spike of orotic acid was caused by endogenous orotic acid in the lysate of HeLa cells (Fig.

16). Thus quantification of produced orotic acid by the enzymatic reaction was readily performed.

The enzymatic production of orotic acid was depending on cell numbers (0 – 3 × 10^5) in the enzymatic reaction mixture (Fig. 17). For the calculation of the specific activity of DHODH in the cells lysate, the incubation time of the enzymatic reaction was set at 1.0 h. The specific activity of DHODH in the present lysate of HeLa cells was 323 pmol/10^5 cells/h.

2.3.4 Specific activities of DHODH in HeLa cells and fibroblast cells

The specific activity of DHODH in fibroblast cells and HeLa cells were measured and compared. The specific activities of DHODH were normalized by numbers of cells involved in the enzymatic reaction mixture. The lysate of HeLa cells and fibroblast cells added to the enzymatic reaction mixture contains equal numbers (3 × 10^5) of cells.

As shown in Fig. 18 A, the enzymatic reaction mixtures containing cell

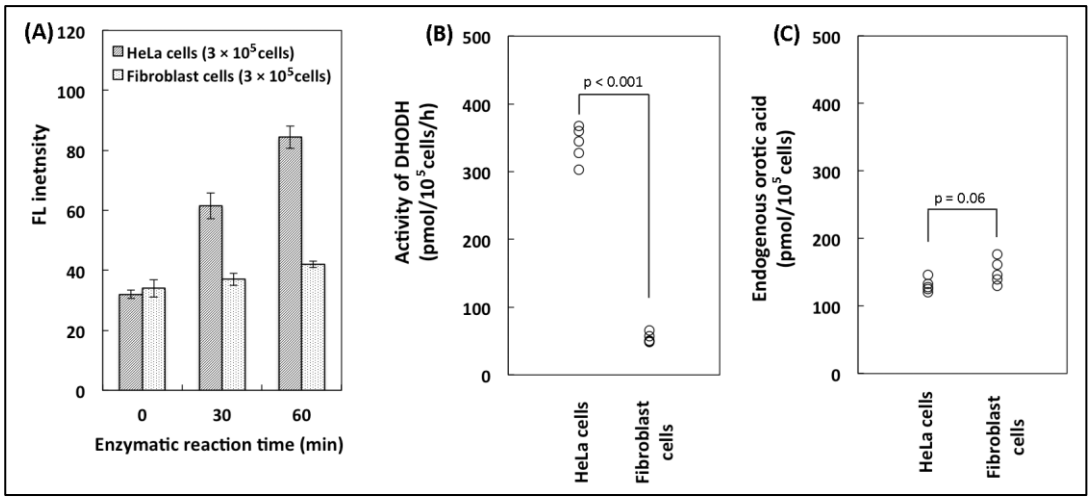


Fig. 18 Comparison of DHODH activities and endogenous concentrations of orotic acid in HeLa cells and fibroblasts.

lysates of either HeLa cells or fibroblast cells were incubated for 30 and 60 min. Both of FL intensities were increased with the enzymatic reaction time at 37 °C. According to the standard curve of FL intensities and concentrations of standard addition of orotic acid to the enzymatic reaction, increased FL intensities (HeLa cells, 30 min: 29.5, 60 min: 52.4; fibroblast cells, 30 min: 3.2, 60 min: 9.8) were

corresponded to concentrations of produced orotic acid (HeLa cells, 30 min: 0.52 μM, 60 min: 0.94 μM; fibroblast cells, 30 min: 0.06 μM, 60 min: 0.17 μM) in the enzymatic reaction mixtures. The data showed DHODH catalyzing the production of orotic acid from the substrate of DHO.

Specific activity of DHODH in HeLa cells and fibroblast cells were calculated and the results were shown in Fig. 18 B. Fig. 18 B showed the day-to-day measurement of specific activities of DHODH in the lysate of HeLa cells and the lysate of fibroblast cell. Specific activity of DHODH in the lysate of HeLa cells (340 ± 25.9 pmol/10^5 cells/h) is approximately 6.3 times higher than that of fibroblast cells (54.1 ± 7.40 pmol/10^5 cells/h). As cancer cells, HeLa cells require adequate pyrimidine concentration for their growth thus HeLa cells have enhanced activities of DHODH than normal cells such as fibroblast cells.

Furthermore, I investigated the endogenous orotic acid in lysate of HeLa cells and fibroblast cells to see if enhancement of DHODH in HeLa cells would cause a higher concentration of endogenous orotic acid. The results were shown in Fig. 18 C, concentrations of orotic acid were 130 ± 9.72 pmol/10^5 HeLa cells and 151 ± 18.6 pmol/10^5 fibroblast cells. The endogenous orotic acid of lysate of HeLa cells and fibroblast cells showed equal levels ($p > 0.05$), suggesting that orotic acid was not increased by the enhancement of activity of DHODH. This is because orotic acid would be rapidly converted to UMP by uridine monophosphate synthetase (UMPS) through the following steps of the de novo biosynthetic pathway. The results agreed with the report that DHODH is a rate-limiting enzyme of the de novo synthetic pathway of pyrimidine[72].

2.3.5 Specific activity of DHODH in lysate of a stomach malignant tumor and its adjacent normal tissue

I investigated the specific activity of DHODH in lysate of a malignant tumor of stomach and in lysate of its adjacent normal stomach tissue. The assay was conducted in the same way as described above. However, normalization of the sample sizes was performed by the measurement of total proteins to avoid the varying weight of wet tissue. Amount of total proteins in the enzymatic reaction mixtures containing the lysates of malignant tumor and lysate of its adjacent normal tissue were both adjusted to 0.75 mg.

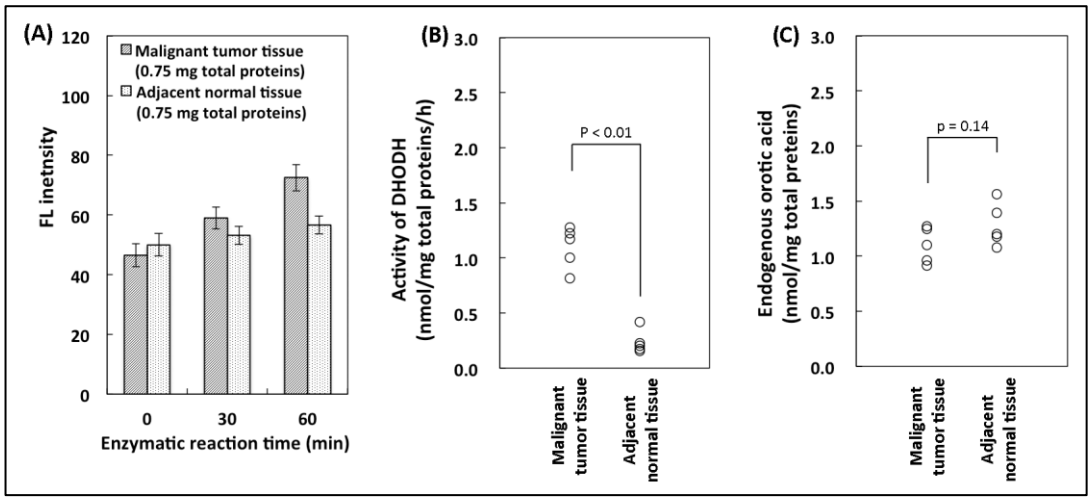


Fig. 19 Comparison of DHODH activities and endogenous concentrations of orotic acid in stomach cancer and adjacent normal tissues.

As shown in Fig. 19 A, both of the enzymatic reaction mixtures were incubated for 30 and 60 min. The increases of the FL intensities (the malignant tumor, 30 min: 10.3, 60 min: 26.5; the normal tissue, 30 min: 1.3, 60 min: 4.2) were corresponded to concentrations of produced orotic acid (the malignant tumor, 30 min: 0.25 μM, 60 min: 0.69 μM; the normal tissue, 30 min: 0.03 μM, 60 min: 0.11 μM) in the enzymatic reaction mixtures.

Fig. 19 B showed the day-to-day measurement (n = 5) of specific activities of DHODH in lysate of the malignant tumor and lysate of its normal tissue. Specific activity of DHODH in the lysate of malignant tumor (1.10 ± 0.19 nmol/mg total protein/h) is approximately 4.6 times higher than that of its adjacent normal tissue (0.24 ± 0.11 nmol/mg total protein/h). The results also suggested that in the malignant tumor, activity of DHODH is s ignificantly higher than that in the

adjacent normal tissue, agreeing with the results of comparison between activity of DHODH in lysate of HeLa cells and fibroblast cells.

Also, concentrations of the endogenous of orotic acid in the malignant tumor tissue and its adjacent normal tissue were determined. The results were shown in Fig. 19 C. The concentration of endogenous orotic acid in the malignant tumor tissue (1.09 ± 0.16 nmol/mg total protein) is not significantly different ($p > 0.05$) from that of the adjacent normal tissue (1.28 ± 0.19 nmol/mg total protein), suggesting that in the malignant tumor, concentration of orotic acid would not be accumulated by the enhanced activity of DHODH.

2.3.6 Colorimetric assay of DHODH using the lysate of HeLa cells as an enzyme source

Fig. 20 showed the principle for the colorimetric assay that has been widely used for screening of effective inhibitors of DHODH. As introduced in the background, dihydroorotate is oxidized by DHODH and releases a pair of electrons, followed by reduction of DCPIP. As a result, DCPIP turns blue to colorless after it was reduced. The absorbance of the enzymatic reaction mixture was measured at 610 nm.

The assay requires purification of DHODH from cell extracts since there are

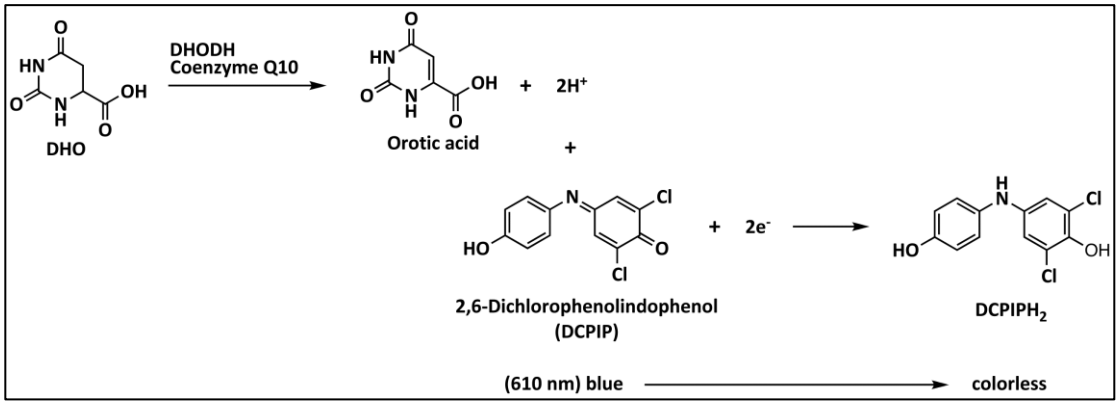


Fig. 20 Principle of colorimetric assay for DHODH.

many co-existing reducing agents that would cause a false positive activity. In Fig. 21, the results of colorimetric assay were shown. The enzymatic reaction was initiated as addition of lysate of HeLa cells.

From the result, it can be seen that absorbance of the reaction mixture decreased equally in the groups of full reaction and blank reaction in which substrate was not added, indicating that the reducing of DCPIP is not due to the enzymatic reaction of DHODH. Thus, the method can be easily disrupted by reducing agents in the lysate thus purification of DHODH should be conducted beforehand. On the contrary, my fluorometric method permits a facile assay for DHODH by using a lysate rather than purification of the enzyme.

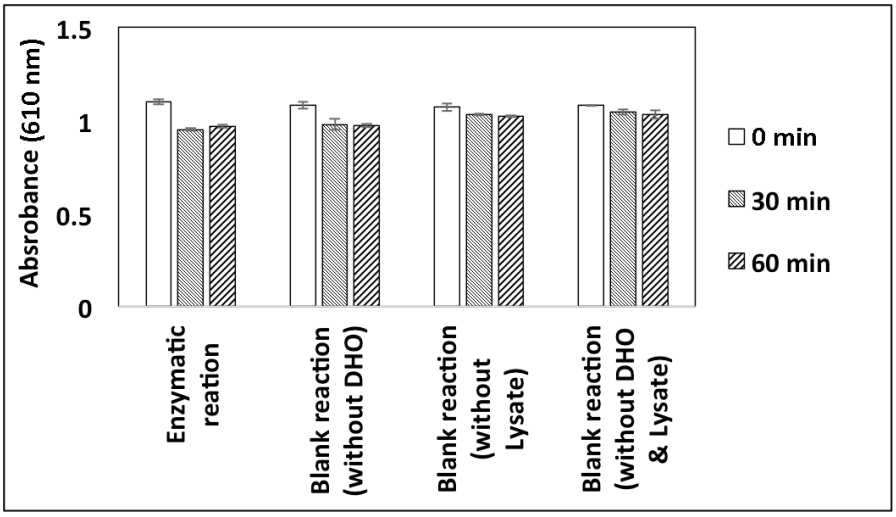


Fig. 21 Colorimetric assay of DHODH using lysate of HeLa cells.

My study firstly reported the activity of DHODH in HeLa cells/fibroblast cells and malignant stomach tumor/its adjacent normal tissue.

2.4 Conclusion

In this study, I have applied the FL reaction for the assay of DHODH activity in lysates of cultured HeLa cells and fibroblast cells and of tissues from malignant stomach tumor and its adjacent normal region from a stage III patient.

Herein I found for the first time that specific activity of DHODH in the cancer HeLa cells is significantly higher than that of the normal fibroblast cells, as well as significantly higher activity of DHODH in malignant tumor tissue than that of its adjacent normal tissue. Thus, DHODH may become a cancer biomarker.

Several inhibitors of the DHODH enzyme have been suggested to have anti-cancer properties and cause apoptosis of the cancer cells[56-59]. However, the activity of DHODH has not been measured in either cultured cells or tissues of human since the convenient assay has not been developed.

On the other hand, my results of quantification of endogenous orotic acid in cultured HeLa cells/fibroblast cells and the malignant tumor tissue/adjacent normal tissue showed that although activity of DHODH is enhanced in cancer cell and tissue, the concentration of orotic acid, the product of DHODH, was not increased. This is because orotic acid would be rapidly converted to UMP by UMPS through the following steps of the de novo synthetic pathway of pyrimidine. These results agreed with the report that DHODH is a rate-limiting enzyme of this pathway[72].

In addition, further study of activity of DHODH in other types of tumors such as the stages of patients, different positions of the tumor of human body remains required. My method would provide a facile conduction of the assay of DHODH as a cancer marker as well as the evaluation of inhibitors for DHODH.

In conclusion, I have provided a novel assay method for the DHODH activity in human tissues. The results suggested a higher activity of DHODH in malignant stomach tumor than its adjacent normal tissue and supported the previous reports that inhibition of DHODH cause apoptosis of cancer cells rather than normal cells. Diagnosis of cancer by the determination of activity of DHODH in a bio-sample using my facile method is highly expected in the further study.

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