J. Biochem. 126, 347-353 (1999)

A New UV Method for Serum γ-Glutamyltransferase Assay Using Recombinant 4-Aminobenzoate Hydroxylase as a Coupling Enzyme

Yukihito Mizutani,*,† Tatsuya Narikawa,*,‡ Takanori Satoh,* Nobuhiko Sakurai,*,§ Hiroyuki Kaji,*,¶ Shingo Yamada,† and Tatsuya Samejima*,‖

*Department of Chemistry, College of Science & Engineering, Aoyama Gakuin University, Setagaya-ku, Tokyo 157-8572; and †Central Institute Shinotest Corporation, Onodai, Sagamihara, Kanagawa 229-0011

Received March 5, 1999; accepted May 28, 1999

4-Aminobenzoate hydroxylase (4ABH) is a flavin-dependent monooxygenase that catalyzes the decarboxylative hydroxylation of 4-aminobenzoate to 4-hydroxyaniline. For use as a clinical reagent, the gene encoding 4ABH from *Agaricus bisporus* was cloned by the RACE method. Also, the cDNA encoding 4ABH was expressed in *Escherichia coli* cells as a fusion protein with glutathione *S*-transferase (GST). The expressed GST-4ABH fusion protein (recombinant 4ABH) in the soluble fraction exhibits decarboxylative hydroxylation and additional NADH oxidation activities. We investigated a new ultraviolet spectrometric method for determining serum γ-glutamyltransferase (γ-GT) using recombinant 4ABH as a coupling enzyme. The principle of the method is as follows. Using γ-glutamyl-3-choloro-4-aminobenzoate (L-γ-glu-PAClBA) and glycylglycine as the donor and acceptor substrates, 3-choloro-4-aminobenzoate (PAClBA) is formed by the catalysis of serum γ-GT. PAClBA is stoichiometrically converted to 3-choloro-4-hydroxyaniline (PHClA) and NAD^+ by 4ABH and NADH. However, NADH oxidation results in a high reagent blank, which is considered as a drawback for use as a clinical reagent. Using recombinant 4ABH, we examined the effects of pH and detergents on these two activities, and found that several detergents suppress the additional NADH oxidation activity with little or no effect on hydroxylation activity. The results indicate a promising approach to establishing an ultraviolet spectrophotometric method for determining serum γ-GT activity using L-γ-glu-PAClBA as the donor substrate and recombinant 4ABH as a coupling enzyme.

Key words: 4-aminobenzoate hydroxylase, γ-glutamyltransferase, γ-glutamyl-3-choloro-4-aminobenzoate, spectrophotometric assay.

γ-Glutamyltransferase (EC 2.3.2.2, γ-GT) catalyzes the transfer of a γ-glutamyl group on a peptide such as glutathione to another peptide or amino acid. This enzyme is the best marker for metastatic invasion of the liver. The clinical importance of this enzyme has been well established (*1-6*), and the methods used recently for measuring serum γ-GT activity have employed either γ-L-glutamyl-3-carboxy-4-nitroanilide (L-γ-glu-3CA4NA) (*7, 8*) or γ-L-glutamyl-4-nitroanilide (L-γ-glu-4NA) (*9*) as the substrate. Because of its solubility, the most widely used substrate is L-γ-glu-3CA4NA, which has been proposed for use in the assay of serum γ-GT activity by IFCC and JSCC. These methods are practicable for routine assay, although there are numerous problems to be solved. We have developed a new method for serum γ-GT determination by ultraviolet spectrometry (UV method) (*10*). This method utilizes 4-aminobenzoate hydroxylase (EC 1.14.13.27, 4ABH) as a coupling enzyme. 4ABH from *Agaricus bisporus*, an edible mushroom, is an external flavoprotein monooxygenase that catalyzes the decarboxylative hydroxylation of 4-aminobenzoate with the consumption of NADH and O_2. It has recently been reported that the molecular mass of 4ABH is 51 kDa and its isoelectric point is 6.1 (*11*). Furthermore, the cloning and sequencing of a cDNA encoding 4ABH have been reported by Tsuji *et al.* (*12*). In addition to the hydroxylation activity, this enzyme also exhibits a very low NADH to NAD^+ oxidation activity in the absence of 4-aminobenzoate (PABA) (*13*). No information has been available concerning the amino acid residues in 4ABH that participate in either the decarboxylative

Present addresses: ‡ Laboratory of Microbial Engineering, Graduate School of Science and Technology, Chiba University Matsudo, Chiba 271-0092; § Division of Materials Science, Institute of Natural Sciences, Nagoya City University, Yamanohata, Mizuho-ku, Nagoya, Aichi 467-8501; and ¶ Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, Minami-osawa, Hachioji, Tokyo 192-0364.

‖ To whom correspondence should be addressed. Tel: +81-3-5384-1111 (Ext. 3204), Fax: +81-3-5384-6200, E-mail: samejima@candy.chem.aoyama.ac.jp

Abbreviations: 4ABH, 4-aminobenzoate hydroxylase; γ-GT, γ-glutamyltransferase; NADH, nicotinamide adenine dinucleotide; L-γ-glu-4NA, L-γ-glu-4-nitroanilide; L-γ-glu-3CA4NA, L-γ-glu-3-carboxy-4-nitroanilide; 3CA4NA, 3-carboxy-4-nitroaniline; L-γ-glu-PAClBA, L-γ-glu-3-choloro-4-aminobenzoate; PAClBA, 3-choloro-4-aminobenzoate; PHClA, 3-choloro-4-hydroxyaniline; 5A2NB, 5-amino-2-nitrobenzoic acid; Glygly, glycylglycine; γ-glu-3,5DCA, γ-glu-3,5-dicarboxyanilide; GST, glutathione *S*-transferase; IFCC, International Federation of Clinical Chemistry; JSCC, Japanese Society of Clinical Chemistry; PABA, *p*-aminobenzoic acid.

hydroxylation or NADH oxidation activities. In order to develop a practicable UV method using 4ABH as a coupling enzyme, two problems need to be resolved. One is the very low yield of 4ABH from the mushroom, and the other is the high reagent blank due to NADH oxidation by 4ABH as a side reaction. Thus, the aim of this work was to clone and express the 4ABH gene in *Escherichia coli* cells first, then investigate the effects of pH and detergents on the decarboxylative hydroxylation and NADH oxidation activities, thereby establishing a new UV spectrometric method for the assay of serum γ-GT activity using 4ABH as a coupling enzyme.

MATERIALS AND METHODS

Materials—Restriction endonucleases were purchased from Nippon Gene, Takara Shuzo, and Toyobo. ISOGEN was purchased from Nippon Gene. A Marathon™ cDNA amplification kit was purchased from CLONTECH. pBluescriptII KS⁻ was purchased from Stratagene. An mRNA purification kit, GST Gene fusion vector pGEX3X, GST Detection Module, Glutathione Sepharose 4B, PBE94, and Polybuffer 74 were purchased from Pharmacia. *Taq* DNA polymerase, DNA Ligation kit ver. 1, alkaline phosphatase from calf intestine, *E. coli* competent cell JM109(DE3), BcaBEST™ Dideoxy Sequencing kit, and FITC-labeled primer were purchased from Takara Shuzo. NADH was obtained from Oriental Yeast. L-γ-glu-PAClBA and glycylglycine (Glygly) were purchased from Kokusan Chemical Works. 4-Aminobenzoate (PABA) was purchased from Tokyo Kasei Organic Chemicals. Isopropyl-β-D-thiogalactopyranoside (IPTG) and FAD were purchased from Boehringer Mannheim. All other chemicals used in this study were obtained from commercial sources and were of the highest grade available. The γ-GT assay kits were obtained from Boehringer Mannheim (Boehringer γ-GT; substrate, L-γ-glu-3CA4NA) and Shinotest (Labomeito γ-GT; substrate, L-γ-glu-3,5-dicarboxyanilide (L-γ-glu-3,5DCA) and Quick auto neo γ-GT JS; substrate, L-γ-glu-3CA4NA). Serum samples were obtained from nearby hospitals. Control serum was purchased from Shinotest (Omega I and II), Azwell (Seraclear N and NA), and International Reagents (Moni-Trol I, II, IIX).

Cloning of 4ABH cDNA—Preparation of total RNA from mushrooms was performed by the AGPC (acid guanidinium thiocyanate-phenol-chloroform) method of Chomczynski *et al.* (*14*). Poly(A)⁺ RNA was separated on a column of oligo(dT)-cellulose (mRNA purification kit), and the mRNA was purified according to the protocol of the mRNA purification kit. The first strand cDNA template was synthesized using poly(A)⁺ RNA prepared from the mushrooms. Also, RACE was performed to obtain 4ABH cDNA using the Marathon™ cDNA amplification kit (*15-19*). The resultant cDNA was digested with *Eco*RI and *Not*I and then subjected to agarose gel electrophoresis. The obtained fragment was subcloned into the same site of pBluescriptII KS⁻. The subcloning vector was confirmed by DNA sequencing.

DNA Sequencing—DNA sequencing was performed with a Hitachi fluorescence automatic DNA sequencer model SQ-3000. The DNA was inserted into the multiple cloning sites of plasmid pUC18 to prepare the sequencing vector. Dideoxy DNA sequencing (*20*) was performed on the denatured plasmid using FITC-labeled primers, which are complementary to sequences flanking restriction sites commonly used for cloning. The elongation reaction was performed by *Bca* BEST™ DNA polymerase.

Expression of the Recombinant 4ABH (GST-4ABH Fusion Protein) in E. coli JM109 (DE3)—Two primers, EP-3 (5′-GGGGATCCATATGTC TCAACAGGAGCGC-3′, sense sequence for amino acid residues 1-6) and EP-4 (5′-CCGGATCCTTATAGCACCGCTCTAGGCC-3′, antisense sequence for residues 454-458), were chemically synthesized for the amplification of cDNA. Each primer was designed for a *Bam*HI restriction site. To construct the 4ABH expression vector, a cDNA fragment containing *Bam*HI sites at both ends was amplified by PCR using primers EP-3 and EP-4. The PCR product was digested with *Bam*HI and then inserted into the same sites, *i.e.*, downstream of the *tac* promoter, of the expression vector pGEX3X (*21*). The constructed plasmid (pGEX4ABH) was transformed into *E. coli* JM109 (DE3). Cells harboring the plasmid were grown in Luria Bertani (LB) medium containing ampicillin (100 μg/ml) at 37°C to an absorbance at 600 nm of 0.5. The cells were then induced by incubation with 0.5 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 5 h at 37°C.

Purification of Recombinant 4ABH from E. coli Cells—Recombinant 4ABH was prepared in a large-scale preparation. First, *E. coli* JM109 (DE3) cells harboring plasmid pGEXABH were cultured in 10 liters of LB medium. After induction with IPTG, the cells were harvested by centrifugation for 10 min at 5,000 rpm. The cells were suspended in 200 ml of phosphate buffer (pH 7.3, 10 mM $NaHPO_4$ and 1.8 mM KH_2PO_4) containing 0.14 M NaCl and 2.7 mM KCl, and lysed by sonication for 1 h on ice. The lysate was centrifuged at 5,000 rpm for 10 min, and the recombinant 4ABH in the soluble fraction was applied directly to a Glutathione Sepharose 4B column pre-equilibrated with the same buffer. The column was washed with the same buffer, and then the recombinant 4ABH was eluted with 300 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM glutathione. The fractions with enzyme activity were collected and concentrated to about 10 ml by ultrafiltration using an Amicon stirred cell with a PM10 membrane. The concentrated fraction was applied to a chromatofocusing column that had been pre-equilibrated with 25 mM imidazole-HCl buffer (pH 7.0) containing 20% glycerol. The recombinant 4ABH was eluted with polybuffer (pH 5.0) containing 20% glycerol. Finally, the collected fraction was applied to a Glutathione Sepharose 4B column to remove the polybuffer used for chromatofocusing. The eluted fractions were concentrated to 1 ml by ultrafiltration using the Amicon PM10 membrane. Thus, the recombinant 4ABH was purified to an electrophoretically homogeneous state.

Assay for Enzyme Activities of the Recombinant 4ABH—The purified recombinant 4ABH was assayed for enzyme activity by determining the amount of indophenol dye (*22*). The reaction mixture contained 0.1 mM of FAD, 0.02% (w/v) bovine serum albumin, 2 mM 4-aminobenzoate (PABA), 0.2 mM NADH, and 50 mM potassium phosphate buffer (pH 7.0) in a total volume of 3 ml. The mixture was preincubated at 30°C for 3 min and the reaction was started by the addition of the enzyme. The mixture was incubated at 30°C for 10 min, and the reaction was stopped

by the addition of 1.2 ml of 20% trichloroacetic acid. The mixture was centrifuged at 3,000 rpm for 10 min, and 0.1 ml of 5% phenol solution containing 2.5 N NaOH and 0.2 ml of 2.5 N Na_2CO_3 was added to 1 ml of supernatant to start the formation of the indophenol dye. The mixture was incubated at 37°C for 1 h, and the absorbance at 630 nm was measured. The apparent Michaelis constants (K_m) of the recombinant 4ABH for 4-aminobenzoate (PABA) were determined by the least squares method on a Lineweaver-Burk plot. The reaction was performed in 50 mM potassium phosphate buffer (pH 7.0 or 8.0) containing 20 μM FAD, 0.02% BSA, 0.2 mM NADH, and 0.1-2.0 mM 4-aminobenzoate (PABA).

NADH oxidation activity was measured spectrophotometrically at 37°C, as the decrease in NADH absorbance at 340 nm over 10 min. One unit of enzyme activity was defined as the oxidation of 1 μmol NADH per minute under the conditions described below. A reaction mixture (500 μl, pH 8.0) containing 50 mM KH_2PO_4, 20 μM FAD, 0.2 mM NADH, and 0.02% BSA was prepared in a cuvette and equilibrated at 37°C for about 5 min. Then 2 μl of recombinant 4ABH was added and mixed by gentle inversion. The absorption decrease at 340 nm was recorded in a spectrophotometer at 37°C for 10 min and ⊿absorbance/min was calculated from the linear portion of the curve. To characterize the recombinant 4ABH, the pH dependence of the hydroxylation and NADH oxidation activities was examined between pH 6.0 and 9.0. Each pH 6.0-9.0 reaction buffer contained 0.2 mM NADH, 0.02 mM FAD, 0.02% (w/v) BSA, and 2 mM 4-aminobenzoate (PABA) in 50 mM sodium phosphate buffer. A Bio-Rad protein assay kit (Bio-Rad Laboratories) with bovine serum albumin as the standard was used to determine the protein concentration of each sample.

SDS-Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis (12%, w/v) with 0.1% SDS (SDS-PAGE) was carried out as described by Laemmli (*23*). The molecular mass was calibrated using myosin (212,000), α2-macroglobulin (170,000), β-galactosidase (116,000), transferrin (76,000), and glutamic dehydrogenase (53,000) as standards. The proteins were stained with Coomassie Brilliant Blue (CBB).

Western Blot Analysis—A cell lysate harboring the recombinant 4ABH was prepared from *E. coli* JM109-(DE3) and boiled in SDS-PAGE loading buffer. The prepared sample was subjected to 12% (w/v) SDS-polyacrylamide gel electrophoresis. The proteins were transferred electrophoretically to nitrocellulose membranes. The nonspecific binding sites were blocked with 1% (w/v) BSA containing 10 mM Na_2HPO_4 and 0.85% (w/v) NaCl, and the membrane was incubated with polyclonal anti-GST antibody for 2 h at room temperature. Immunoreactive proteins were visualized with peroxidase-conjugated anti-(mouse IgG) antibody.

Assay of γ-GT Activity in Serum by the JSCC Method—The measurement of serum γ-GT activity is based on the principles developed by Orlowski and Meister (*9*) and Shaw *et al.* (*7, 8*). The measurements were carried out with a Hitachi 7150 automatic analyzer. The γ-GT assay was done using two-separate reagents, Reagent I (RI), Glygly buffer (pH 8.2), and reagent II (RII), L-γ-glu-3CA4NA in acetic acid (pH 5.2). L-γ-Glu-3CA4NA and Glygly are used as substrates for the reactions catalyzed by human serum γ-GT. The activity was measured by recording the absorbance change at 410 nm, the absorption wavelength of 5-amino-2-nitrobenzoic acid (5A2NB; the cleavage product of the donor substrate). The reaction is carried out by incubating 10 μl of serum with 250 μl of RI at 37°C for 5 min, and then adding 125 μl of RII. After an 84 s lag period, the absorbance is measured for 3 min at 410 nm and the γ-GT activity is calculated by the following equation.

$$\text{L-}\gamma\text{-glu-3CA4NA} + \text{Glygly} \xrightarrow{\gamma\text{-GT}} \text{L-}\gamma\text{-glu-Glygly} + \text{5A2NB}$$

The Effect of Detergent on Serum γ-GT Activity—The effect of detergents on serum γ-GT activity was examined using a γ-GT assay kit (Quick auto neoγ-GT JS; substrate, L-γ-glu-3CA4NA). Serum γ-GT activity was measured in samples after detergent was added to RI. The detergents used for suppressing NADH oxidation activity were obtained from NIKKOL as follows: four non-ionic detergents, BT-9 (polyoxiethylene [9] alkylether), decaglyn 1-IS (monoisostearate decaglycerin), BL-9EX (polyoxyethylene [9] laurylether), and TAMNO-15 (polyoxyethylene [15] oleylamine); an amphoteric detergent Anhitoru 24B (betaine ampholytic surfactant), and two anionic detergents, SLS (sodium laurylsulfate) and SBL-2N-27 (polyoxyethylene [2] sodium laurylethersulfate). The hydroxylation and NADH oxidation activities of the recombinant 4ABH were examined in the presence of 0.1% of each detergent.

Assay of γ-GT Activity in Serum by a New UV Method—The procedure for the assay of γ-GT by a new UV method is as follows (*10*). Measurements were carried out with a Hitachi 705 automatic analyzer using two-separate reagents, reagent I (RI) and reagent II (RII). RI contained 50 mM potassium phosphate buffer (pH 8.0), 100 mM Glygly, 0.28 mM NADH, 28 μM FAD, 0.028% BSA, and 0.1% Anhitoru 24B; RII contained 50 mM potassium phosphate buffer (pH 8.0), 50 mM L-γ-glu-PAClBA, 100 mM Glygly, and 4ABH. The assay was carried out at 37°C. A 50 μl serum sample was added to 400 μl of RI, and then 100 μl of RII was added to start the reaction. The absorbance at 340 nm was monitored continuously with both a Hitachi 705 Automatic Analyzer and a Hitachi 220A Spectrophotometer. A reagent blank consisted of the same mixture with 50 μl of distilled water in place of the serum.

The serum γ-GT activity was calculated from the linear portion of the curve after a lag phase. The principle of this method is based on the following reaction: serum γ-GT liberates PAClBA in the transfer reaction from L-γ-glu-PAClBA to γ-glutamyl acceptor-Glygly. The released PAClBA is converted to 3-choloro-4-hydroxyaniline (PHClA) by 4ABH. Thus, the PAClBA produced by γ-GT action is stoichiometrically converted to PHClA by 4ABH forming NAD^+ from NADH.

$$\text{L-}\gamma\text{-glu-PAClBA} + \text{Glygly} \xrightarrow{\gamma\text{-GT}} \text{L-}\gamma\text{-glu-Glygly} + \text{PAClBA}$$

$$\text{PAClBA} + \text{NADH} \xrightarrow{\text{4ABH}} \text{PHClA} + \text{NAD}^+$$

RESULTS

cDNA Cloning of 4ABH—The constructed plasmid vector containing the complete cDNA of 4ABH had an open

reading frame of 1,380 nucleotides encoding for 460 amino acids. The molecular mass of 4ABH was estimated to be 51 kDa from the deduced amino acid sequence, coinciding completely with the report of Tsuji *et al.* (*13*). This perfect coincidence indicates that the reliability and reproducibility of their results and that the reported nucleotide and amino acid sequences of 4-ABH are genuine.

Expression of the Recombinant 4ABH in E. coli—A cell lysate harboring the expressed recombinant 4ABH was analyzed by 12% SDS-PAGE. We confirmed that the recombinant 4ABH is expressed in the soluble fraction of the cells. Furthermore, immunoblot analysis using anti-GST antibody showed the recombinant 4ABH corresponding to a molecular mass of 77 kDa is immunologically positive. Thus, we succeeded in overexpressing recombinant 4ABH in *E. coli* as a fusion protein with glutathione-*S*-transferase (GST).

Purification of the Recombinant 4ABH from E. coli—The result of the purification of recombinant 4ABH is summarized in Table I. The enzyme was purified to 30 times the activity of the crude extract with a 39.4% yield. By this purification, 3.67 mg of pure recombinant 4ABH was obtained from the transformed *E. coli* JM109(DE3) cells in 10 liters of LB medium. The hydroxylation activity was measured using this purified enzyme and the specific activity was found to be 0.91 unit/mg protein. The expressed recombinant fusion protein was used to develop the UV method for serum γ-GT assay and to examine the suppression of NADH oxidation activity.

pH Dependence of the Enzyme Activities of the Recom-

TABLE I. **Purification of the recombinant 4-aminobenzoate hydroxylase [glutathione-*S*-transferase (GST)]-[4ABH fusion protein].**

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Crude extract	253	8.46	0.03	100	1
Glutathione Sepharose 4B	56.7	31.8	0.56	379	19
Chromatofocusing	11.5	9.18	0.80	109	27
Glutathione Sepharose 4B	3.67	3.33	0.91	39.4	30

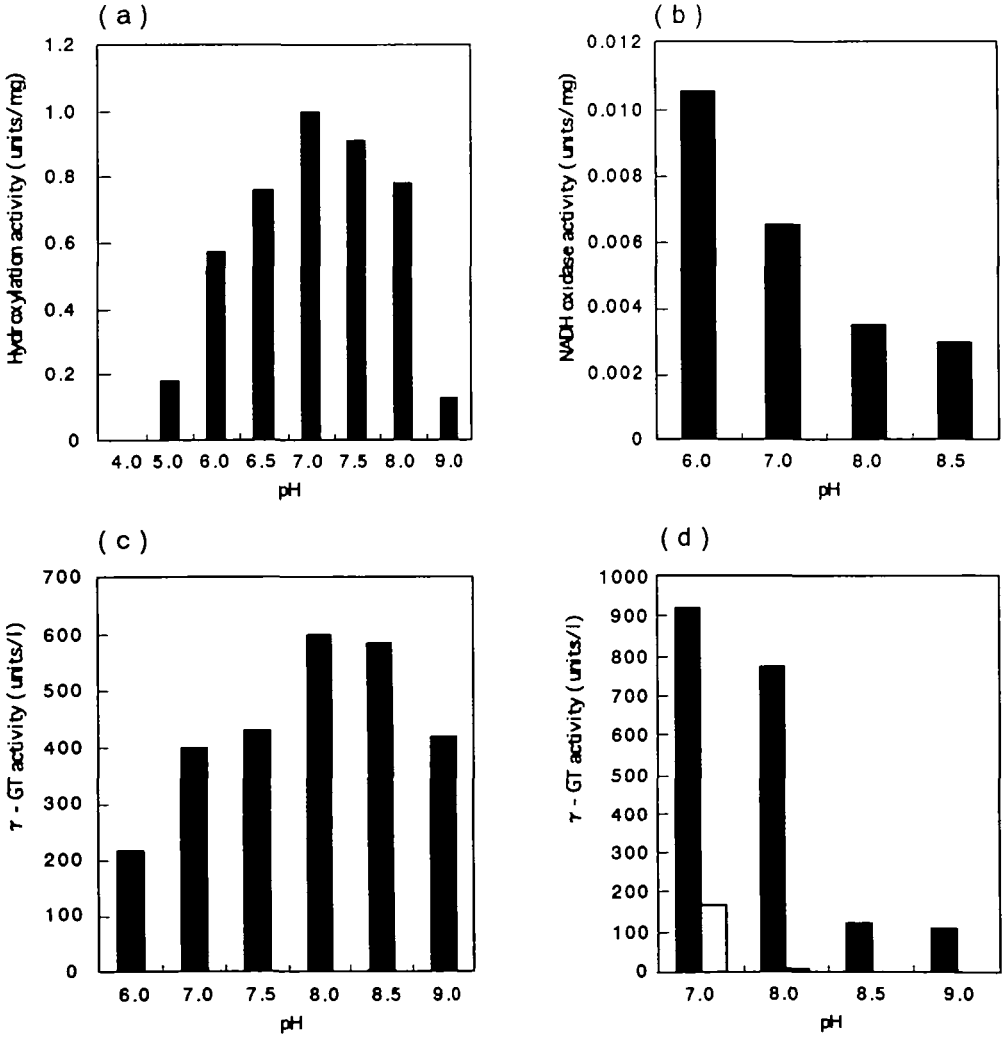

Fig. 1. **pH dependence of the decarboxylative hydroxylation activity, the NADH oxidation of the GST-4-aminobenzoate hydroxylase fusion protein (recombinant 4ABH), and the γ-GT activity.** (a) Effect of pH on the hydroxylation activity of recombinant 4ABH. (b) Effect of pH on the NADH oxidation activity of recombinant 4ABH. (c) Effect of pH on γ-GT activity of serum. (d) Effect of pH on the reagent blank and γ-GT activity on the UV method. □: reagent blank, ■: γ-GT activity. Experimental conditions are described in "MATERIALS AND METHODS."

binant 4ABH—To characterize the recombinant 4ABH we first examined the pH dependence of the hydroxylation and NADH oxidation activties. The apparent K_m values of the recombinant 4ABH for 4-aminobenzoate (PABA) were 111.7 μM at pH 7.0 and 473.9 μM at pH 8.0. This indicates that the substrate affinity is about four times less at pH 8 than at neutral pH. Also, the recombinant 4ABH showed its maximal hydroxylation activity at pH 7.0 (Fig. 1a). The recombinant 4ABH also exhibited an additional weak NADH oxidation activity as a side reaction with a pH dependence as shown in Fig. 1b. We found that the NADH oxidation activity was minimum at pH 8.0 in the pH range examined. The catalytic concentration of γ-GT in serum was examined by a quick auto neoγ-GT JS kit (Shinotest). The pH dependence of the γ-GT activity in serum was measured at 37°C between pH 6.0 and 9.0, with an optimum pH observed at pH 8.0 (Fig. 1c). Moreover, the pH dependence of the new UV assay method was also investigated at 37°C between pH 7.0 and 9.0. The relation between the reagent blank and γ-GT activity in this pH range is shown in Fig. 1d. These results indicate that the optimum pH of this assay method using recombinant 4ABH is about 8.0. At this pH the hydroxylation activity of 4ABH is mostly retained, while the NADH oxidase activity is mostly suppressed when recombinant 4ABH is used.

Suppression of the Reagent Blank by Detergent—Next, the effect of several detergents on these activities was examined in order to suppress the NADH oxidation activity, which caused a high reagent blank. Several non-ionic and amphoteric detergents produced significant suppression of the NADH oxidation activity, although they had differing effects on the activities of hydroxylation and serum γ-GT. The non-ionic detergents produced a significant suppression of NADH oxidase activity, while Anhitoru 24B had a considerable inhibitory effect, but caused a slight suppression of the hydroxylation activity. However, the anionic detergents completely inhibited hydroxylation and a significant reduction in the γ-GT activity (Table II).

Establishment of the UV Method for Measuring Serum γ-GT Activity—We investigated the UV method for measuring serum γ-GT activity using L-γ-glu-PAClBA as a substrate and purified recombinant 4ABH as a coupling enzyme. L-γ-Glu-PAClBA was found to be the most specific and sensitive donor substrate in a previous study (*10*). The time course in the γ-GT assay was linear after a lag phase of about 1 min. Distilled water as a reagent blank was assayed with the addition of Anhitoru 24B. The addition of detergent above 0.01% diminished the reagent blank almost completely as shown in Fig. 2. In this case, the reagent blank is converted to the corresponding value for

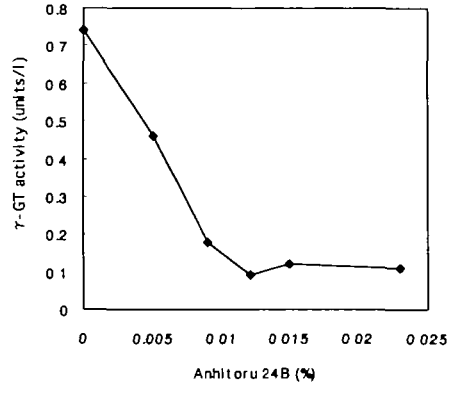

Fig. 2. **Effect of Anhitoru 24B concentration on the reagent blank for the UV method for the assay of serum γ-GT activity.** The absorbance decrease at 340 nm by NADH oxidation was converted to the corresponding γ-GT activity on the ordinate.

TABLE II. **Effect of detergents on the NADH oxidation, hydroxylation, and γ-GT activities.**

Detergent	Classification	NADH oxidation activity (×10⁻² units/mg)	(%)	Hydroxylation activity (units/mg)	(%)	Serum γ-GT activity (units/l)	(%)
Control	—	1.30	100	1.50	100	60.0	100
BT-9	Non-ionic	0.43	33.0	1.50	100	60.0	100
TAMNO-15	Non-ionic	0.49	38.0	1.61	107	61.8	103
Decagln1-IS	Non-ionic	0.16	12.0	1.62	108	60.0	100
BL-9EX	Non-ionic	0.34	26.0	1.61	107	61.2	102
Anhitoru 24B	Amphoteric	0.46	35.0	1.05	70.0	60.0	100
SBL-2N-27	Anionic	0.58	45.0	0.00	0.00	58.8	98.0
SLS	Anionic	0.40	31.0	0.00	0.00	7.20	12.0

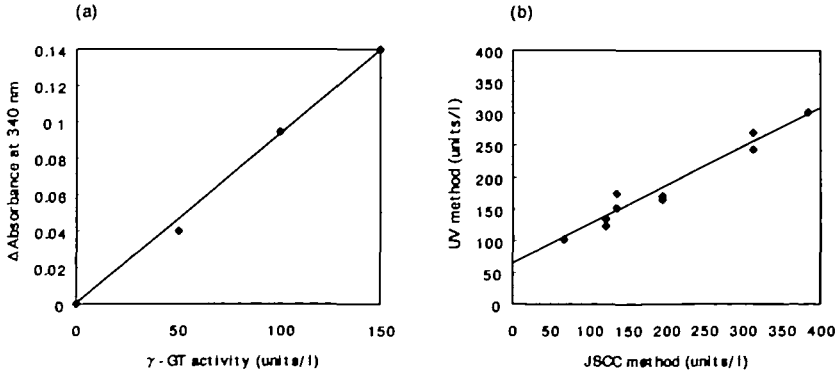

Fig. 3. **The evaluation of the UV method using recombinant 4ABH.** (a) Relationship between the absorbance at 340 nm determined by the UV method and γ-GT activity. (b) Correlation between the JSCC method and the UV method. The activity measurements were performed under the conditions described in "MATERIALS AND METHODS." The correlation coefficient was 0.988 and the regression equation for the data was $y = 0.64x$.

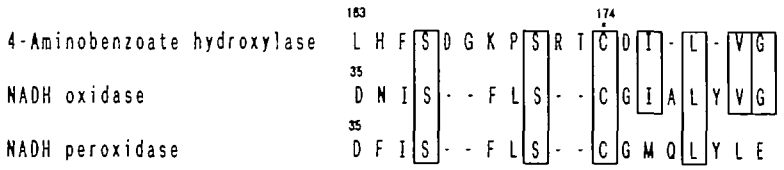

Fig. 4. **Comparison of the amino acid sequence of a segment of 4-aminobenzoate hydroxylase with those of conserved redox-active segments of NADH oxidase and NADH peroxidase.** Homologous residues are boxed and gaps are shown as dashes. The deduced redox-active cysteinyl residues are indicated by an asterisk.

serum γ-GT activity. Anhitoru 24B suppresses the NADH oxidation of 4ABH, therefore, the reagent blank is remarkably suppressed. Serum γ-GT activity was also determined by this new UV method using Anhitoru 24B to suppress the NADH oxidation. The linearity of the γ-GT activity from the absorbance at 340 nm by this method is illustrated in Fig. 3a.

Comparison of the UV Method with the JSCC Method—γ-GT activity was assayed in 10 serum samples by this new UV method and the standard JSCC method. Figure 3b shows a good correlation between the two methods (correlation coefficient 0.988).

DISCUSSION

The recommended IFCC and JSCC methods for the determination of serum γ-GT activity are now used for routine assay. Although numerous methods have been reported, many problems remain to be solved. For example, L-γ-glu-4NA shows poor solubility and degradation, and the absorption spectrum of L-γ-glu-3CA4NA overlaps with the maximum absorbance of 5A2NB formed by γ-GT action. Moreover, these methods are influenced by colored substances such as hemoglobin and bilirubin present in serum. Therefore, we tried to develop a UV method for the determination of serum γ-GT using authentic 4ABH from *Agaricus bisporus* (*10*). However, there are two serious problems that make this approach very difficult. One is the very low yield of 4ABH prepared from the mushroom, and the other is the side reaction of 4ABH, namely, NADH oxidation, which causes a causing high reagent blank. This method cannot be adopted for practical use unless these problems can be overcome. Therefore, in this study we aimed to evaluate recombinant 4ABH as a coupling enzyme in the UV method for the determination of serum γ-GT activity.

First, in order to establish a large-scale expression system for 4ABH in *E. coli*, the cDNA of 4ABH was cloned by the RACE method (*15-19*). The expression vector of 4ABH gene was constructed using the *tac* promoter and GST gene fusion system (*21*), and a large amount of active GST-4ABH fusion protein (recombinant 4ABH) was produced in *E. coli* transformed cells. The expressed 4ABH was present predominantly in the soluble fraction of the cell extract. The purification by Glutathione Sepharose 4B affinity chromatography and chromatofocusing provided recombinant 4ABH at a moderate or high yield. However, the specific activity of the recombinant 4ABH was lower than that reported for authentic 4ABH (*11*), which may be due to the effect of the GST moiety as a fusion protein. Neverthless, these results suggest that the recombinant 4ABH is expressed in an active form in the *E. coli* cells, and that the inserted DNA fragment includes the intact gene encoding the functional hydroxylase.

Second, the NADH oxidation activity was examined. Similar to authentic 4ABH, the recombinant 4ABH also showed NADH oxidation activity. Although NADH oxidase has been isolated and purified from various sources, the activity of the recombinant 4ABH was compared with that of the enzyme from an extreme thermophile, *Thermus acquaticus* (*24*). The result showed that the NADH oxidation activity of the thermophile is much higher than that of the recombinant 4ABH. We found that the optimum pH for the hydroxylation activity is 7.0 and that for NADH oxidation is about 6, but it shows minimum activity at pH 8-8.5. Also, the optimum pH for serum γ-GT activity is commonly pH 8.0, as also detected in this work. At pH 8.0, the hydroxylation activity was mostly retained, while the NADH oxidation activity was considerably suppressed by several detergents. On the new UV assay system, the reagent blank at pH 7.0 was high and γ-GT activity at pH 8.5 and 9.0 were very low. On the other hand, the reagent blank was suppressed and γ-GT activity was retained at pH 8.0. Therefore, we conclude that the optimum pH for the serum γ-GT assay using the recombinant 4ABH is about 8.0 (Fig. 1 and Table II). Fortunately, this coincidence in the pH optima for these enzymes encouraged us to develop the UV method. If the NADH oxidation activity can be completely suppressed at pH 8.0 without a remarkable reduction in the hydroxylation activity, this method might be promising for practical use. To suppress the NADH oxidation activity efficiently, we examined the effect of detergents on the activity at pH 8.0. The results revealed that several detergents suppress the NADH oxidation activity at this pH. These potent detergents include non-ionic detergents such as BT-9, decaglyn 1-IS, BL-9EX, and TAMNO-15, and an amphoteric detergent, Anhitoru 24B (Table II). However, Anhitoru 24B had a slight effect on the hydroxylation activity at pH 8.0. After examining the suppression of the NADH oxidation activity, we examined the UV method for determining serum γ-GT activity using Anhitoru 24B. The results showed that among the detergents examined, only Anhitoru 24B can be used because the other detergents showed very poor time courses of measurement. From this finding, it was deduced that non-ionic detergents may cause a change in the environment or conformation of NADH itself by surrounding or binding to NADH, thus altering its spectrophotometric characteristics. However, there was no apparent conformational change in 4ABH caused by the non-ionic detergents because the concentrations employed were very low. Although Anhitoru 24B had a slight effect on the hydroxylation activity, we determined the γ-GT activity by this assay system using Anhitoru 24B (Figs. 2 and 3). The effect of Anhitoru 24B on 4ABH could be explained by either some small conformational change or some subtle change in a functional group on 4ABH. It was considered that the absorbance of NADH and the quantitative estimation of the UV method are unaffected by these subtle changes, although we have no definite evidence for this interpreta-

tion so far. We assume that the active sites of these two activities may presumably be located independently in 4ABH.

As described above, we obtained the recombinant 4ABH from the *E. coli* cells in moderate yield, and the reagent blank by the UV method was diminished significantly by Anhitoru 24B. Furthermore, this method correlates well with the JSCC method. Also, this UV method was found to be potentially unaffected by interfering substances in serum, such as hemoglobin and bilirubin. Therefore, we have the possibility that this UV assay will be of practical use. Meanwhile, we noticed that a cysteine residue in the redox-active site of NADH oxidase is also conserved in 4ABH. Therefore, the peptide segment containing the cysteine residue in 4ABH was compared with those of some NADH oxidases as shown in Fig. 4. Comparing the amino acid sequence of 4ABH with those of NADH oxidases (*25-28*), we assumed that Cys174 in 4ABH may correspond to the active site for NADH oxidation. It is likely that these amino acid residues, including Cys174 in 4ABH, may be responsible for the switching or modulating of the two functional activities. Further detailed experiments are needed in order to elucidate the genuine active site in 4ABH and the mechanisms for the decarboxylative hydroxylation and NADH oxidation. Such experiments, including the preparation and characterization of Cys174-substituted mutants and other mutants, are now in progress. Our goal is to suppress the NADH oxidation activity more completely and to establish the UV method by improving the function of 4ABH by protein engineering.

We thank Professor Hideaki Tsuji of Okayama Prefectural University and Professor Yasuo Natori of Tokushima University for their kind advice and discussion. We also thank Shinotest Co. for financial support of this study.

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